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THE SIMILARITY BETWEEN ANEMIA INDUCED BY HYPOPHYSECTOMY AND THAT INDUCED BY A COMBINED THYROIDECTOMY AND ADRENALECTOMY IN ADULT FEMALE RATS[1]

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THE mechanism by which removal of the hypophysis induces an anemia (see reviews by Gordon and Charipper, 1947, and Daughaday, Williams and Daland, 1948) is still unsolved. Thyroidectomy in rats has been shown to induce an anemia which was not as severe as that induced by hypophysectomy (Crafts, 1941; Wilson, 1944; Gordon, Kadow, Finkelstein and Charipper, 1946). Adrenalectomy in rats has been shown to induce an anemia 10 to 20 days after removal of the glands. This anemia, however, gradually disappeared. Sixty days after adrenalectomy, the blood picture was normal (Crafts, 1941; Piliero, Landau and Gordon, 1950; Gordon, Piliero and Landau, 1951).

Many different therapies have been utilized in an attempt to prevent the anemia which develops following hypophysectomy. Those which have been completely effective are pituitary extracts (Flaks, Himmel and Zlotnik, 1938; Contopoulos, Van Dyke, Simpson, Garcia, Huff, Williams and Evans 1953), a combination of thyroxine, testosterone propionate, and a high protein diet (Crafts, 1949), and cobalt nitrate (Crafts, 1952; Garcia, Van Dyke and Berlin, 1952).

The present experiment is a comparison between changes induced in the

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blood picture of the rat by hypophysectomy and that induced by a combined thyroidectomy and adrenalectomy.

METHODS

Adult female rats of 3–4 months of age and of the Wistar strain were used in these experiments. All rats were fed standard Purina chow *ad libitum* supplemented once a week by lettuce. The completeness of all surgical operations was checked by examination of the organ sites at autopsy with a binocular dissecting microscope. Blood was obtained by anesthetizing the rat with ether and clipping the tail. Five or 6 drops of blood were placed in a small vial containing a minute amount of heparin. Erythrocyte counts and white cell counts were done in duplicate using U. S. certified blood pipettes and the improved Neubauer counting chamber. Hemoglobin determinations were made in a Klett-Summerson photoelectric colorimeter. Hematocrit determinations were made with Van Allen hematocrit tubes (no diluent) and spun for 1 hour at 2000 r.p.m. Bone marrows were treated in the following manner:—femurs were removed from the animal, both ends cut off, and one side of the shaft removed. This left the bone marrow in a "boat" of bone which was placed in Helly fixative. After washing, the tissue was placed in 70% alcohol. At this point in the procedure, the marrow was removed from the bone in a long strip. This was sectioned at 6 micra and stained with Giemsa stain.

RESULTS

This experiment includes data on 61 rats. Fourteen served as normal controls, 16 were hypophysectomized, 12 were thyroidectomized, 11 were adrenalectomized, and 8 were thyroidectomized and adrenalectomized. All animals were studied for 60 days after surgery, blood being examined at 10 day intervals. Data on erythrocyte counts are presented in Figure 1; hematocrit readings, Figure 2; and on hemoglobin levels, Figure 3. Values for mean corpuscular volume (M.C.V.), mean corpuscular hemoglobin (M.C.H.), and mean corpuscular hemoglobin concentration (M.C.H.C.) are presented in Table 1. Observations on the white count and bone marrow are included in the text.

Normal Controls

No significant changes occurred in the blood picture of the normal control rats during the 60 days of observation. The blood picture of these rats before the experiment started was as follows:—erythrocyte count, 8.56 ± 0.56[3] million cells per cu. mm.; hematocrit reading, $45.7 \pm 3.2\%$; hemoglobin, 16.0 ± 0.9 gm. per 100 cc. of blood; mean corpuscular volume, 52.0 ± 2.4 cubic microns; mean corpuscular hemoglobin, 18.0 ± 1.2 micromicrograms; mean corpuscular hemoglobin concentration, $34.7 \pm 1.9\%$. On the 60th day of the experiment, these rats exhibited the following blood picture:—erythrocyte count, 8.53 ± 0.54 million cells per cu. mm.; hematocrit

[3] Standard Deviation.

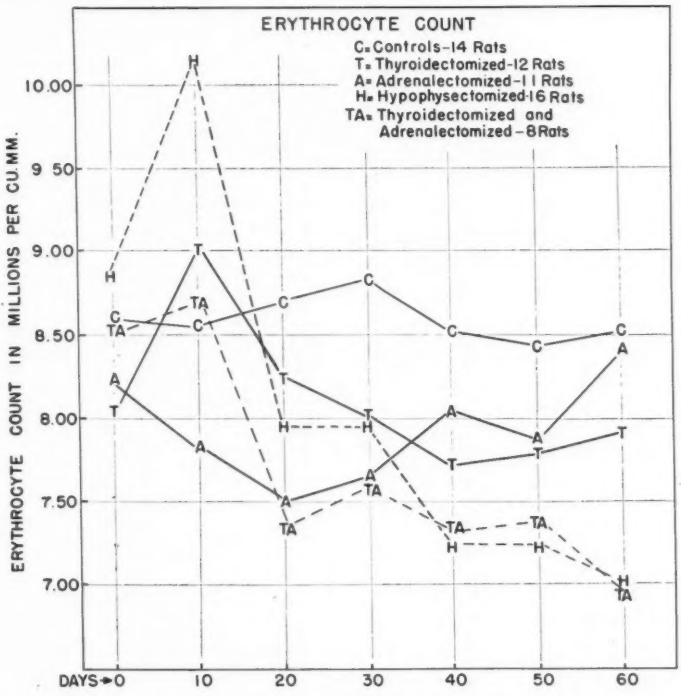

FIG. 1. The effects of thyroidectomy, adrenalectomy, hypophysectomy, and of combined thyroidectomy and adrenalectomy on the erythrocyte count of adult female rats.

reading, 46.0±0.2%; hemoglobin, 16.4±1.2 gm. per 100 cc. of blood; mean corpuscular volume, 54.0±2.3 cubic microns; mean corpuscular hemoglobin, 19.2±1.6 micromicrograms; mean corpuscular hemoglobin concentration, 35.5±2.4%. The total white cell count, on the 60th day of observation, averaged 11.6±3.8 thousand cells per cu. mm. The bone marrows of these control rats were normal in appearance. The body weight increased from an average of 210 gm. before the experiment began to 248 gm. 60 days later.

Thyroidectomy

Removal of the thyroid gland induced a decrease in hematocrit reading from 45.2±3.8% before thyroidectomy to 39.5±1.8% on the 60th day after thyroidectomy. The hemoglobin level in these rats decreased from 15.7±1.4 gm. per 100 cc. of blood to 14.3±0.7 gm. during the 60 days of observation. The erythrocyte count did not exhibit a decrease. The low average erythrocyte count for this particular group of rats before thyroid-

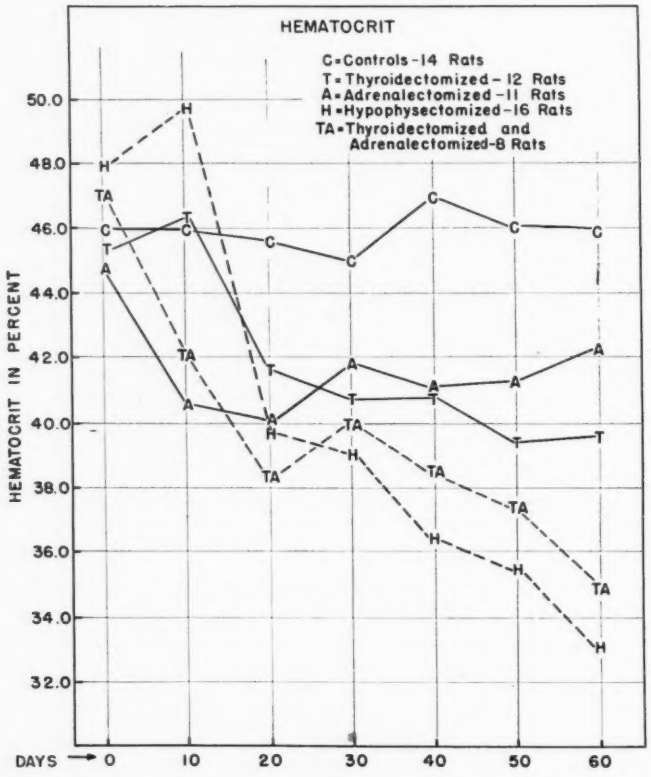

FIG. 2. The effects of thyroidectomy, adrenalectomy, hypophysectomy, and of combined thyroidectomy and adrenalectomy on the hematocrit of adult female rats.

ectomy was performed (Fig. 1) may be the explanation. On the 60th day of observation, the erythrocyte count was 7.97 ± 0.50 million cells per cu. mm. as compared to 8.53 ± 0.54 million cells for the normal controls on this same day. Thyroidectomy induced a decrease in mean corpuscular volume from 54.9 ± 3.7 cubic microns before surgery to 49.4 ± 3.4 cubic microns on the 60th day of the experiment. This is a statistically significant difference. There were no significant changes in mean corpuscular hemoglobin or in mean corpuscular hemoglobin concentration (Table 1). The total white cell count in these thyroidectomized rats averaged 15.7 ± 5.7 thousand cells per cu. mm. on the 60th day of the experiment. The bone marrows of these rats contained a greater proportion of fat than those of the normal control rats, and there was a decrease in number of erythroid elements. The body weight decreased from an average of 203

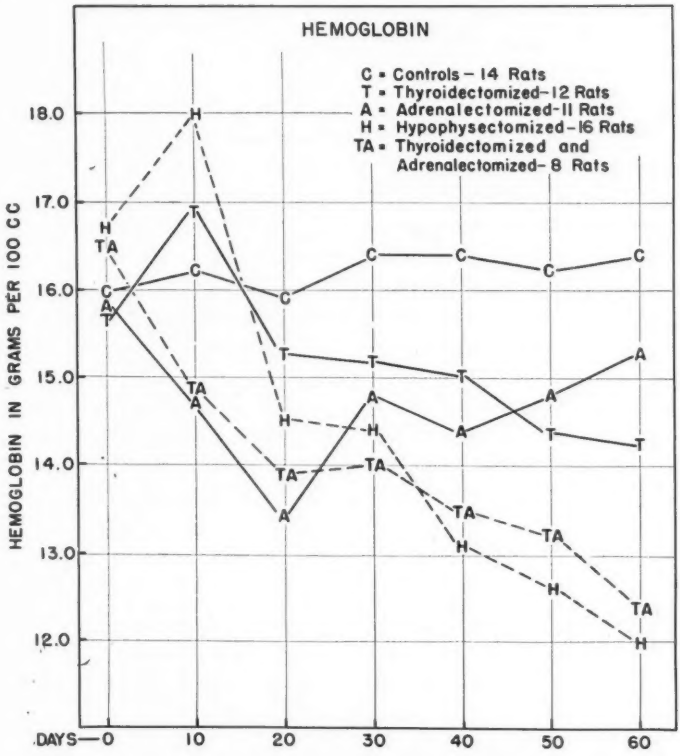

FIG. 3. The effects of thyroidectomy, adrenalectomy, hypophysectomy, and of combined thyroidectomy and adrenalectomy on the hemoglobin of adult female rats.

gm. before thyroidcetomy to 186 gm. 60 days after removal of this gland.

Adrenalectomy

Removal of the adrenals (rats given 1% NaCl as drinking water) induced a temporary anemia. During the first 20 days after adrenalectomy, the erythrocyte count decreased from 8.22 ± 0.50 million cells per cu. mm. before adrenalectomy to 7.50 ± 0.77 million cells; the hematocrit reading, from $44.8 \pm 2.5\%$ to $40.0 \pm 2.8\%$; and the hemoglobin level, from 15.8 ± 0.5 gm. per 100 cc. to 13.4 ± 0.4 gm. During the subsequent 40 days, the blood picture gradually returned to normal. On the 60th day of observation the erythrocyte count was 8.43 ± 0.49 million cells per cu. mm., the hematocrit reading $42.3 \pm 4.5\%$, and the hemoglobin level 15.3 ± 1.5 gm. per 100 cc. of blood. Adrenalectomy did not induce significant decreases in mean corpuscular volume, mean corpuscular hemoglobin, or

TABLE 1. THE EFFECTS OF THYROIDECTOMY, ADRENALECTOMY, HYPOPHYSECTOMY, AND A COMBINED THYROIDECTOMY AND ADRENALECTOMY ON THE MEAN CORPUSCULAR VOLUME (M.C.V.), MEAN CORPUSCULAR HEMOGLOBIN (M.C.H.), AND MEAN CORPUSCULAR HEMOGLOBIN CONCENTRATION (M.C.H.C.) OF ADULT FEMALE RATS

Treatment	Day	M.C.V. in cubic microns	M.C.H. in micro-micrograms	M.C.H.C. in per cent
Normal Controls—No Treatments—14 rats	0	52.0 ±2.4	18.0 ±1.2	34.7 ±1.9
	60	54.0 ±2.3	19.2 ±1.6	35.5 ±2.4
		$P = >0.1$	$P = >0.1$	$P = >0.1$
Thyroidectomized—12 rats	0	54.9 ±3.7	18.8 ±1.2	34.4 ±1.3
	60	49.4 ±3.4	17.8 ±1.1	35.9 ±3.4
		$P = <0.02$	$P = >0.1$	$P = >0.1$
Adrenalectomized—11 rats	0	53.4 ±3.6	18.4 ±1.1	34.4 ±3.0
	60	50.5 ±5.0	18.1 ±2.0	35.8 ±2.7
		$P = >0.1$	$P = >0.1$	$P = >0.1$
Hypophysectomized—16 rats	0	52.8 ±3.4	18.5 ±0.6	35.1 ±1.7
	60	47.6 ±2.9	17.0 ±1.8	35.5 ±1.7
		$P = <0.01$	$P = <0.05$	$P = >0.1$
Thyroidectomized—Adrenalectomized—8 rats	0	53.4 ±3.2	18.9 ±1.5	35.0 ±2.6
	60	50.2 ±1.7	17.4 ±3.0	35.2 ±1.9
		$P = >0.1$	$P = >0.1$	$P = >0.1$

± = Standard Deviation.
P = Fisher's "t" test of significance.

mean corpuscular hemoglobin concentration (Table 1). On the 60th day of the experiment the total white cell count of these adrenalectomized rats averaged 15.4±2.7 thousand cells per cu. mm. The bone marrows of the adrenalectomized rats were quite different than those of the thyroidectomized animals. The amount of fat was decreased and the number of erythroid elements was normal in 7 of the 10 marrows studied. The remaining 3 marrows exhibited a decrease in erythroid elements. The body weight increased from an average of 195 gm. before adrenalectomy to 216 gm. 60 days after adrenalectomy.

Hypophysectomy

Removal of the hypophysis induced the usual anemia. The erythrocyte count decreased from 8.78 ± 0.4 million cells per cu. mm. before hypophysectomy to 7.13 ± 0.4 million cells 60 days later; the hematocrit reading, from 48.0 ± 2.6% to 33.4 ± 0.2%; and the hemoglobin, from 16.8 ± 0.7 gm. per 100 cc. of blood to 12.0 ± 0.9 gm. Hypophysectomy induced a significant decrease in mean corpuscular volume from 52.8 ± 3.4 cubic microns before hypophysectomy to 47.6 ± 2.9 cubic microns 60 days later. This decrease in erythrocyte size induced a slight but significant decrease in mean corpuscular hemoglobin from 18.5 ± 0.6 micromicrograms before hypophysectomy to 17.0 ± 1.8 micromicrograms on the 60th day of the experiment. The mean corpuscular hemoglobin concentration did not change (Table 1). The hypophysectomized animals exhibited a total white cell count of 15.7 ± 5.7 thousand cells per cu. mm. on the 60th day of the experiment. Hypophysectomy induced an increase in marrow fat and a decrease in the number of erythroid elements. These animals decreased in weight from an average of 204 gm. before hypophysectomy to 181 gm. on day 60 of the study.

Thyroidectomy and Adrenalectomy

The removal of both the thyroid and adrenal glands (animals given 1% NaCl as drinking water) induced an anemia which duplicated that which followed hypophysectomy. The erythrocyte count decreased from a preoperative level of 8.54 ± 0.7 million cells per cu. mm. to 7.03 ± 0.4 million cells 60 days later (hyp.—7.13 ± 0.4); the hematocrit reading, from 47.4 ± 1.5% to 35.1 ± 1.3% (hyp.—33.4 ± 0.2); and the hemoglobin level, from 16.7 ± 0.7 gm. per 100 cc. to 12.4 ± 0.9 gm. (hyp.—12.0 ± 0.9). Although the changes in mean corpuscular volume and mean corpuscular hemoglobin in these thyroidectomized-adrenalectomized rats are not statistically significant (Table 1), they are similar to those decreases found in the hypophysectomized rats. The mean corpuscular hemoglobin concentration was not altered by the dual surgery. On the 60th day of the experiment the total white cell count in the thyroidectomized-adrenalectomized animals averaged 16.1 ± 2.9 thousand cells per cu. mm. (hyp.—15.7 ± 5.7). A comparison between the bone marrows of the hypophysectomized and the thyroidectomized-adrenalectomized groups of animals showed that 13 of 14 bone marrows studied in the hypophysectomized group exhibited a decrease in erythroid elements and 5 out of 7 bone marrows studied in the thyroidectomized-adrenalectomized group exhibited this decrease. The body weight decreased from a preoperative average of 209 gm. to 194 gm. 60 days later.

DISCUSSION

The anemia induced by hypophysectomy in this experiment is similar to that reported many times in the literature, but differs in a less severe decrease in erythrocyte number. In a previous publication (Crafts, 1949) a 30% decrease in erythrocyte count was reported after hypophysectomy in the Long-Evans strain of rats. The present work shows a decrease in erythrocyte count from 8.78 to 7.13 million cells per cu. mm. This is only an 18.7% decrease. This work, however, was done with Wistar rats and is the same decrease as previously found with this Wistar strain of rats (Crafts, 1952).

A combined thyroidectomy and adrenalectomy has induced an anemia in these rats which duplicates that induced by hypophysectomy. The fact that neither thyroidectomy nor adrenalectomy alone induced anemia of such severity brings up the point of the mechanisms by which this combined surgery interferes with erythropoiesis. Simply adding the anemia induced by thyroidectomy to that induced by adrenalectomy does not produce an anemia of the severity of that induced by the combined surgery or by hypophysectomy. Work is now in progress in an attempt to determine why this combined surgery produces such an anemia.

It is interesting to recall the study of Flaks, Himmel, and Zlotnik (1938) in which they reported that a preparation of beef anterior pituitary, which was free of thyrotropic, gonadotropic, and growth hormones, would prevent the anemia which was induced by hypophysectomy. The extract was given to rats orally. These authors introduced an "erythropoietic factor" in the pituitary. Recently Contopoulos, Van Dyke, Simpson, Garcia, Huff, Williams and Evans (1953) repeated the above work and could not obtain similar results with beef pituitary extract, but did substantiate Flaks and co-workers with sheep pituitary extract. This extract did not stimulate the thyroid or the gonads but did stimulate the adrenal cortex to a slight extent. Repeating this work on adrenalectomized rats and obtaining similar results have led Contopoulos and co-workers to agree with the conclusion that there is an erythropoietic factor secreted by the pituitary gland. Thus, we have the confusing picture of one set of data indicating that the thyroid and the adrenals are not necessarily involved in the anemia which follows hypophysectomy and the data presented in this paper indicating that the removal of these glands induces an anemia which mimics the anemia induced by hypophysectomy.

It should be noted that the beef pituitary extracts prepared by Flaks and co-workers and the sheep pituitary extracts prepared by Contopoulos and co-workers were given to hypophysectomized rats orally. Assuming such an "erythropoietic factor" would have a similar chemical nature as

other pituitary hormones, it is difficult to see why it should be effective when administered by this route.

The mechanisms by which hypophysectomy induces an anemia have not been determined. However, in rats, removal of both the thyroid and the adrenal glands induces an anemia which mimics that induced by hypophysectomy. The anemia which follows hypophysectomy may be due to a combination of hypothyroidism and hypoadrenocorticalism.

SUMMARY

Sixty-one adult female rats of the Wistar strain were divided into 5 groups and received the following treatment—(1) normal controls—no treatment, (2) thyroidectomized, (3) adrenalectomized, (4) hypophysectomized, and (5) thyroidectomized and adrenalectomized. The blood picture was studied in each group. Thyroidectomy induced a moderate anemia and adrenalectomy a temporary anemia. Hypophysectomy induced an anemia which was duplicated by removal of both the thyroid and the adrenals. These data indicate that there is a possibility that the anemia which is induced by hypophysectomy is due to a combined hypothyroidism and hypoadrenocorticalism.

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COMPARISON OF THE EPITHELIAL PERCENTAGE AND NUCLEAR VOLUME DETERMINATIONS AS INDICATORS OF THYROID ACTIVITY FOLLOWING STIMULATION BY THYROTROPIN[1]

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A NUMBER of methods, both direct and indirect, have been developed for the demonstration of thyrotropin. The direct methods, employed chiefly for the quantitative assay of the hormone, are based on various changes produced in the thyroid gland by thyrotropin (TSH), whereas the indirect methods are concerned with the changes produced by the increased secretion of thyroxin. For a survey of the various methods presented in the literature I refer to my earlier study on the subject (Tala, 1952).

In the same publication I reported my own studies on bioassays of thyrotropin, made by observing the quantitative histological changes in the thyroid epithelium, colloid and stroma of young male guinea pigs produced by thyroid-stimulating hormone (TSH) and thyroid hormone (TH). The linear measurement method presented by Uotila and Kannas (1952) for determination of the percentages of these three components of the thyroid gland was used in these experiments. The percentage of epithelium (E%) was found by me to be the most suitable indicator, when this method is used, of the histological changes produced by these hormones. In one of the test series in the study the E% values were compared with values obtained by measurement of the height of the epithelial cells. Under the conditions of the experiments the two methods were found to be approximately equal in accuracy.

In another study (Kannas and Tala, 1953) the linear measurement method, which had proved very suitable for experimental studies of the thyroid gland, was compared with determinations of the volume of epithelial cell nuclei. The guinea pigs were treated in various ways with TSH,

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iodine and thiouracil. The results obtained by the two methods were found to correspond approximately, for when the epithelial percentage on the abscissa was plotted against the nuclear volume on the ordinate a straight line was obtained.

In the present study a comparison was made of the serviceability of the epithelial percentage and nuclear volume determinations for use as indicators of thyroid activity after stimulation of the gland with small doses of TSH.

MATERIAL AND METHODS

The comparative study was made on the series of test animals autopsied on June 27, 1951, as described in the first investigation referred to above (Tala, 1952). The test animals were young male guinea pigs of as uniform weight as possible. The animals grew under identical conditions of light, heat and diet. The individuals selected for the experiment were kept in a room with a window, at a temperature of $20 \pm 2°$ C. They were fed on hay, Swedish turnips and oats.

Stimulation of the thyroid was effected with powdered TSH, 1 mg. of which contained about 13 Junkmann-Schoeller (JS) guinea pig units of TSH. The substance was dissolved and serial dilutions containing 1, 0.5, 0.02, 0.001 and 0.00001 JS unit per ml. were made in physiological saline (0.9%). The guinea pigs were divided into six groups of ten animals each, one group forming the controls. Members of each of the other groups received a given dilution of TSH by subcutaneous injection of 1 ml. of the dilution into the animal's back, this method of application appearing to be the least strenuous for the animal. The controls received at the same time 1 ml. of physiological saline. The animals were killed 2 hours after the injection by decapitation, which was considered to be the most rapid method and the least harmful to the thyroid gland. Both lobes of the thyroid were dissected out carefully, cleaned, and fixed immediately in Bouin's fluid for 6 hours to prevent postmortal changes. After fixation the specimens were treated with rising concentrations of alcohol. The lobes were embedded in paraffin, sectioned longitudinally with the microtome into two halves and 8–10 sections were cut at 3 μ from the median surfaces. Staining was by Koneff's modification (1938) of the Mallory-Azan method, as previously described (Tala, 1952).

The degree of activity of the thyroid gland was determined by the above mentioned quantitative histological method of Uotila and Kannas (1952) for the calculation of the percentages of different components of the thyroid gland. In this case the percentage of epithelium (E%) was calculated. The sections cut from the median portion of the thyroid were mounted on slides and placed in an apparatus projecting the image in powerful magnification downward onto a white plane by which the investigator was seated. The apparatus constructed by Oy. G. W. Berg, Helsinki, and the method of measurement have been described earlier (Tala, 1952).

The measurements of cell nuclei were made according to the principles presented by Jakobj (1931). A monocular microscope giving a magnification of 2,600 times and furnished with a Zeiss-Abbe drafting apparatus was used for tracing the circumference of the nuclei. Drawing the same nucleus twice was precluded by the use of a cross-ruled table. Calottes and decomposing or atrophied nuclei were discarded. Only the homogeneously stained and approximately round (difference in the length of diameters not over 16%) nuclear rings in the center of the field of vision were drawn. The nuclear

measurements were made from the same sections as the E% measurements. Several investigators, as for instance Ludwig (1950) and Wilflingseder (1947), consider the measurement of 50 nuclei adequate when the variations in nuclear size are small. In the present work, also, 50 nuclei were measured from the left lobe of the thyroid gland.

The nuclear diameter was obtained by adding the length of the longest diameter and that of the diameter at right angles to it, and dividing the sum by 2 (the nuclei were nearly spherical). The mean nuclear diameter in μ and the mean nuclear volume in μ^3 were calculated for each animal. In order to obtain a single figure for comparison with the E%, the average mean nuclear volume for each group of test animals was calculated and compared with the average change in the percentage of epithelium in the same group.

RESULTS

The epithelial percentages (E%) are given in Table 1, which also shows the changes in the percentage of epithelium (ΔE%), obtained by subtracting the average percentage of epithelium in the thyroids of the control

TABLE 1. PERCENTAGE OF EPITHELIUM (E%) AND INCREASE IN PERCENTAGE OF EPITHELIUM OVER AVERAGE E% OF CONTROLS (ΔE%) IN GUINEA PIG THYROIDS AFTER ACTIVATION BY DIFFERENT AMOUNTS OF TSH. TEN ANIMALS IN EACH DOSAGE GROUP

Controls		Dose of TSH in JS units									
		1		0.5		0.02		0.001		0.00001	
E%	E%	E%	ΔE%	E%	ΔE%	E%	ΔE%	E%	ΔE%	E%	ΔE%
63.5	69.4	87.0	20.1	81.9	15.0	76.2	9.3	73.8	6.9	73.1	6.2
70.2	62.2	80.9	14.0	79.9	13.0	76.7	9.8	72.8	5.9	73.0	6.1
62.5	71.6	83.2	16.3	78.5	11.6	74.8	7.9	74.1	7.2	71.5	4.6
64.9	70.0	75.8	8.9	83.0	16.1	73.3	6.4	71.7	4.8	74.8	7.9
66.8	57.4	76.5	9.6	72.7	5.8	77.6	10.7	77.5	10.6	73.4	6.5
66.3	77.4	80.4	13.5	79.0	12.1	72.7	5.8	73.1	6.2	68.6	1.7
70.2	76.0	83.8	16.9	71.1	4.2	72.9	6.0	78.5	11.6	73.7	6.8
65.7	74.1	79.6	12.7	80.6	13.7	74.0	7.1	72.0	5.1	71.7	4.8
61.0	58.0	78.0	11.1	83.3	16.4	79.5	12.6	70.3	3.4	68.8	1.9
69.4	61.2	87.4	20.5	72.4	5.5	73.1	6.2	69.8	2.9	78.1	11.2
Average	66.9	81.3	14.4	78.2	11.3	75.1	8.2	73.4	6.5	72.7	5.8

animals from the percentage of epithelium in the thyroid of each treated animal. Table 2 shows the various nuclear diameters and the average nuclear volume in the control group and each test group. Figure 1 depicts graphically the results obtained by the two methods of measurement following stimulation of the thyroid gland with TSH.

For comparison of the linear measurement and nuclear volume methods, the average change in E% on the abscissa was plotted against the average nuclear volume on the ordinate. As shown in Figure 2, the averages fall on a nearly straight line, which proves that the results obtained by the two methods are analogous.

DISCUSSION

Using the percentage of epithelium as the indicator of thyroid activation, I was able to demonstrate in the earlier study (Tala, 1952) by the

TABLE 2. NUCLEAR DIAMETERS AND AVERAGE NUCLEAR VOLUME IN GUINEA PIG THYROIDS AFTER ACTIVATION BY DIFFERENT AMOUNTS OF TSH. TEN ANIMALS IN EACH DOSAGE GROUP

Nuclear diameter (μ)	Controls	Dose of TSH in JS units				
		1	0.5	0.02	0.001	0.00001
4.50	1					
4.75	2					3
5.00	28					3
5.25	47		1		2	10
5.50	218		1	4	17	40
5.75	118	2	5	14	42	45
6.00	33	2	6	26	56	51
6.25	52	20	49	110	131	100
6.50	1	34	30	89	86	51
6.75		63	78	116	99	64
7.00		80	95	97	40	50
7.25		48	54	21	14	23
7.50		46	67	18	10	7
7.75		30	46	5	1	2
8.00		58	43		2	1
8.25		32	11			
8.50		22	4			
8.75		28	7			
9.00		9	2			
9.25		8	0			
9.50		11	0			
9.75		4	1			
10.00		3				
Average nuclear volume in μ^3	93.6	233.0	196.2	154.1	140.3	135.9

linear measurement method the small amount of 0.00001 unit of TSH This result was confirmed in the same study by tests in which the animals were pretreated for 3 days with TH and given TSH on the 3 following days.

In the present study, the same results were confirmed by another method, the nuclear volume measurement method. In other words, it was possible to demonstrate 0.00001 JS unit of TSH by both methods and to obtain almost identical curves for the increasing doses. To my knowledge there are no reports in the literature on the demonstration of equally low concentrations by the nuclear volume method.

In considering the relative merits of the two methods it should be noted that a single section of the thyroid will give by the linear measurement method an accurate picture of the proportion of epithelium in the thyroid in relation to the other components, as the two intersecting lines along which the measurements are made extend nearly to the periphery of the section. On the other hand, if an exact and dependable value is to be obtained by the nuclear volume measurements it is necessary to measure a vast number of nuclei in both the center and the periphery of the gland. In the latter case a certain degree of selection will be unavoidable and as a consequence the possibility of subjective error is greater in this method than in the epithelial percentage method. The disadvantages of the nuclear

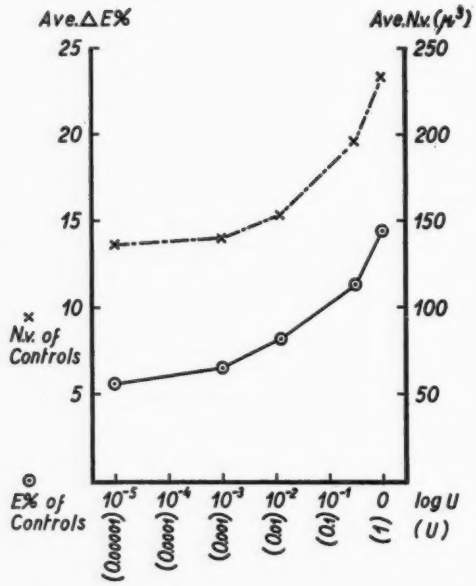

FIG. 1. Relationship between change in average percentage of epithelium, average nuclear volume, and dose of TSH, using a response time of 2 hours. Ave. $\Delta E\%$ = change in average percentage of epithelium (solid line). Ave. N. v. (μ^3) = average nuclear volume in μ^3 (broken line). U = dose of TSH.

volume measurements are therefore the same as those of the cell height measurements. It should furthermore be noted that determination of the nuclear volume requires considerably more time than the determination of the epithelial percentage.

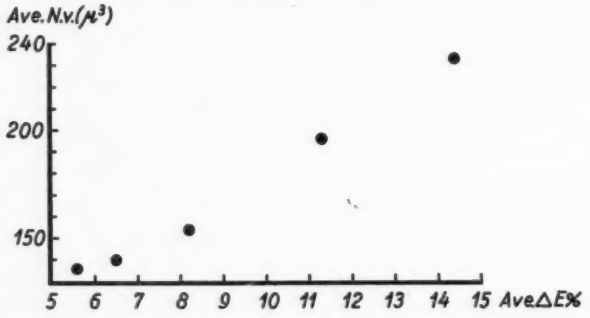

FIG. 2. Comparison of the results obtained by the linear measurement method and the nuclear volume method. Ave. $\Delta E\%$ = change in average percentage of epithelium. Ave. N. v. (μ^3) = average nuclear volume in μ^3.

In making a choice between these two methods, which give practically analogous results, the epithelial percentage method appears preferable in view of its facility and greater dependability. The question comes to mind whether changes in the epithelial percentage and the nuclear volume are not two different aspects of the same phenomenon, since they appear to be parallel. They both are evidently indicators of the activation of the epithelial cell.

SUMMARY

Comparisons were made of the results obtained by the methods of measuring the epithelial percentage and the nuclear volume as indicators of thyroid activity following stimulation of the thyroid of young male guinea pigs by small amounts of TSH. It was found that both methods of measurement were able to detect 0.00001 JS unit of TSH and gave practically identical graphic curves. However, the linear measurement method of determining the percentage of epithelium in the thyroid gland was considered more objective and rapid.

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THE EFFECT OF INJECTIONS OF TESTOSTERONE PROPIONATE AND CORTISONE ACETATE ON GLYCOGEN DEPLETION INDUCED BY EPINEPHRINE IN DIFFERENT SKELETAL MUSCLES[1]

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IT HAS been shown that under a wide variety of conditions the following hormones increase the deposition of glycogen in the skeletal muscles of the rat; insulin (Bridge, 1938); growth hormone (Illingworth and Russell, 1951); testosterone (Leonard, 1952; Bowman, 1953); adrenal cortical hormones (Long, Katzin and Fry, 1940; Leonard, 1953); and estradiol (Leonard, 1953). Injection of epinephrine, on the other hand, rapidly depletes muscle glycogen which subsequently returns to normal (Cori and Cori, 1928; Strand and Gordon, 1952). Therefore the question arises whether or not the glycogenolytic effects of epinephrine on muscle glycogen will be altered if the animals are pretreated with one of the above glycopexic hormones. In this study, only testosterone and cortisone were employed to elevate the muscle glycogen levels prior to epinephrine treatment. Since there are marked differences in the ability of a number of skeletal muscles to store glycogen under the influence of these steroids (Leonard, 1952, 1953; Bowman, 1953), it was considered essential to use several different skeletal muscles in this investigation.

METHODS

Adult female rats of the Long-Evans strain, weighing from 180–220 grams were used. Within a given experiment, the animals were selected for uniformity of age and weight. Subcutaneous injections of 1 mg. testosterone propionate[2] in peanut oil, or 2 mg. of cortisone acetate[2] in a water suspension were administered daily for three days, prior to autopsy. The controls received injections of the same amount of oil or water. Epinephrine was injected intraperitoneally in doses of 5 μg. per 100 gm. body weight in the experiments in which the rats were treated with testosterone and cortisone. This dose of

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epinephrine was chosen as the result of a number of experiments which will be presented in detail later.

In all experiments, the rats were starved for 24 hours preceding the injection of epinephrine. The muscles were removed from each rat exactly 10 minutes following an intraperitoneal injection of sodium pentobarbital (6 mg. per 100 gm. body weight).

The *rectus femoris*, abdominal and diaphragm muscles were removed and analyzed for glycogen content by methods previously described (Leonard, 1952). The glycogen was determined by the anthrone method (Seifter *et al.*, 1950), and is reported as mg. of glucose per 100 gm. of muscle, wet weight.

RESULTS

Determination of the dose of epinephrine.—It was necessary to establish an optimal dose of epinephrine to meet certain requirements for this investigation. First, the dose must be sufficient to significantly lower the glycogen in all three muscles in one hour; this time was selected in order to permit the employment of a reasonable number of animals in an experiment. Secondly, the dose must be small enough so that a serious and pro-

TABLE 1. EFFECT OF EPINEPHRINE ON GLYCOGEN DEPLETION IN SKELETAL MUSCLE

Experiment†		Glycogen (mg./100 gms. of muscle)*					
Epineph.	Period	Rectus femoris		Abdominal		Diaphragm	
(μg.)	(hours)	Exper.	Control	Exper.	Control	Exper.	Control
2.5	½	276 ± 21	346 ± 16	*203 ± 24*	381 ± 20	*120 ± 11*	247 ± 18
2.5	1	371 ± 21	349 ± 17	*301 ± 16*	371 ± 22	215 ± 12	232 ± 8
5.0	1	*261 ± 20*	349 ± 17	*178 ± 22*	371 ± 22	*152 ± 15*	232 ± 8
5.0	2	355 ± 23	357 ± 15	238 ± 27	279 ± 19	*338 ± 16*	269 ± 14
5.0	3	348 ± 10	357 ± 15	258 ± 16	279 ± 19	*372 ± 16*	269 ± 14

* The averages are based on results of 7–11 animals in each experiment and are presented with the standard error of the mean. Numbers in italics are significantly different from their controls, $p < .02$.

† Epinephrine given as a single injection and muscles removed after designated period.

longed upset in the carbohydrate metabolism of the animals would not likely occur. It was arbitrarily decided that if the glycogen levels of the muscles returned to normal within two to three hours, this last condition would be reasonably fulfilled. Preliminary tests indicated that the optimal dose would fall between 2.5 and 5 μg. of epinephrine per 100 gm. body weight (the latter determined on the day the food was removed). To establish the effect of these doses on a quantitative basis, a large number of female rats were injected intraperitoneally with 2.5 and 5 μg. of epinephrine per 100 grams body weight and autopsied one-half, one, two, and three hours later. The results are presented in Table 1.

It was observed that the 5 μg. dose of epinephrine significantly lowered the glycogen levels in all three muscles in one hour. When the 2.5 μg. dose was injected, a significant decrease in the level of muscle glycogen was

noted only in the abdominal muscle at the end of one hour. However, the 2.5 μg. dose was sufficient to decrease the glycogen content in all muscles in one-half hour, but since this period was too brief for convenience, the larger dose was used in the remaining experiments.

When the 5 μg. dose of epinephrine was given, the glycogen of the rectus femoris and abdominal muslces returned to a level which was not significantly different from that of the controls within two hours. In the diaphragm, however, not only did the glycogen level return to normal, but it also increased significantly above the initial level. There was no further change in glycogen levels between the second and third hours after injection. When 2.5 μg. of epinephrine were injected and the muscles examined one hour afterwards, the glycogen levels of the rectus femoris and diaphragm muscles were restored to normal, whereas that of the abdominal muscle was still significantly low.

It was noted that the net decrease in glycogen, as a result of injecting epinephrine, was greatest in the abdominal muscle and that the restoration of glycogen to the initial level was slower than in the other muscles. The intraperitoneal injections of epinephrine induced a severe straining and contraction of the abdominal muscles of the rats which, in itself, might have contributed to the marked lowering of glycogen and the subsequent slower return to normal. It was found, however, that these abdominal contractions could be eliminated if the rats were under anesthesia throughout the period of epinephrine action. In order to determine the effect on the glycogen levels, 6 rats, having been anesthetized for 10 minutes, were injected with the 5 μg. doses of epinephrine and were autopsied one hour later. The controls were anesthetized for a similar period of time.

It was observed that the net loss of glycogen in all three muscles in this experiment was greater than that which occurred when the same amount of epinephrine was given before anesthesia. The net loss for the rectus femoris, abdominal and diaphragm muscles was 139, 263, 199 mg. %, respectively for the rats given the anesthetic first, as compared to 88, 193, 80 mg. %, respectively, for the animals given epinephrine first. However, since the decrease in glycogen in the abdominal muscle in the absence of straining was greater than that in the other muscles, it was assumed that the abdominal contractions were not contributing to the marked glycogenolytic effect of epinephrine in this muscle.

Effect of testosterone propionate.—When normal female rats were injected with 1 mg. of testosterone propionate per day for 3 days, a statistically significant increase in glycogen was observed in the rectus femoris, abdominal, and diaphragm muscles (Table 2). It was also noted that the net increase in glycogen induced by this steroid was greatest in the abdominal muscles.

In order to determine the glycogenolytic effect of epinephrine on the

TABLE 2. ELEVATION OF MUSCLE GLYCOGEN BY TESTOSTERONE PROPIONATE AND CORTISONE ACETATE

Experiment	Glycogen (mg./100 gms. of muscle)*					
	Rectus femoris		Abdominal		Diaphragm	
	Exper.	Control	Exper.	Control	Exper.	Control
Testosterone pr.	*478 ± 19*	366 ± 20	*551 ± 35*	396 ± 15	*357 ± 20*	293 ± 15
Cortisone ac.	*519 ± 23*	417 ± 12	*613 ± 22*	524 ± 14	358 ± 11	337 ± 14

* Same as Table 1.

muscles of rats treated with testosterone, a group of animals were injected with testosterone as described above, and one hour before autopsy, they were given 5 μg. doses of epinephrine. The results are presented in Table 3. Although it was observed that the initial glycogen levels were higher in the testosterone treated animals, the net loss of glycogen in each muscle induced by the injection of epinephrine was approximately the same as that in the corresponding muscle of the untreated rats. Since the effect of the epinephrine did not appear to be altered by previous treatment with this steroid, no experiments were conducted to determine the rate of restoration of depleted glycogen in the testosterone treated animals.

Effect of cortisone acetate.—The results of injecting normal female rats with 2 mg. of cortisone acetate per day for 3 days are presented in Table 2. The data indicates that cortisone increased the glycogen levels in the rectus femoris and abdominal muscles but failed to do so in the diaphragm. The net increase in glycogen was greatest in the rectus femoris muscle.

When epinephrine was injected into rats pretreated with cortisone, the glycogenolytic effect was evident in the abdominal and diaphragm muscles but completely absent in the rectus femoris muscle (Table 3). The net loss

TABLE 3. EFFECT OF CORTISONE ACETATE AND TESTOSTERONE PROPIONATE ON GLYCOGEN DEPLETION WITH EPINEPHRINE

Muscle*	Glycogen (mg./100 gm. muscle)†					
	Epinephrine		Epineph. & Testost.		Epineph. & Cortisone	
	Exper.	Control	Exper.	Control	Exper.	Control
Rectus femoris	*261 ± 20* (−88)‡	349 ± 17	*472 ± 15* (−64)	536 ± 12	507 ± 18 (−6)	513 ± 30
Abdominal	*178 ± 22* (−193)	371 ± 22	*417 ± 12* (−169)	586 ± 13	*388 ± 21* (−94)	482 ± 15
Diaphragm	*152 ± 15* (−80)	232 ± 8	*309 ± 14* (−121)	430 ± 14	*264 ± 24* (−92)	356 ± 20

* Muscles removed 1 hour after injection of 5 μg. of epinephrine per 100 gms. body weight.

† The averages are based on results of 9–15 animals in each experiment and are presented with the standard error of the mean. Numbers in italics are significantly different from their controls, $p < .01$.

‡ Numbers in ( ) indicate net loss of glycogen.

of glycogen in the abdominal and diaphragm muscles was the same. When the net loss of glycogen in the abdominal muscles of normal rats, induced by injections of epinephrine, is compared with that in the cortisone treated rats, however, a partial inhibition of the glycogenolytic action of epinephrine by cortisone is evident. A similar comparison between the diaphragm muscles indicates no inhibition by cortisone.

An experiment was conducted to determine whether or not the complete and partial inhibition of epinephrine action by cortisone in the rectus femoris and abdominal muscles, respectively, was due either to a delaying of the glycogenolytic response or to an acceleration of the rate of restoration of the depleted glycogen. Groups of rats were injected with cortisone, then given epinephrine at the same dose level, but autopsied one-half hour and two hours later. No depletion of glycogen in the rectus femoris muscle occurred at either time interval. The abdominal muscle showed a net loss of 101 mg. % of glycogen in one half hour but had returned to normal in two hours. A net loss of 157 mg. % occurred in the diaphragm but the glycogen also returned to normal in two hours. These results indicated that the effect of cortisone was that of actually inhibiting the breakdown of glycogen by epinephrine, rather than delaying the glycogenolytic response or accelerating the rate of restoration of the depleted glycogen.

It can be demonstrated, however, that larger doses of epinephrine will overcome the complete inhibitory action of cortisone in the rectus femoris muscle. Five cortisone treated rats were injected with 20 μg. of epinephrine per 100 grams body weight and the rectus femoris muscle was removed one half hour after the injection. The net loss of glycogen induced by this large dose of epinephrine, acting for a period of one half hour, amounted to 201 mg. %.

DISCUSSION

The results of these experiments indicate a marked difference in the ability of testosterone propionate and cortisone acetate to influence the glycogenolytic effect of epinephrine in skeletal muscle. The net decrease in glycogen in the rectus femoris, abdominal and diaphragm muscles observed in the normal female rat was unaffected by prior treatment of the animals with doses of testosterone propionate sufficient to significantly increase the glycogen levels in these muscles. Previous administration of cortisone acetate, however, completely inhibited the glycogenolytic effect of epinephrine in the rectus femoris muscle, partially inhibited it in the abdominal muscles, but failed to inhibit it in the diaphragm. While this work was in progress, Winternitz and Long (1952) reported the antagonistic effect of adrenal cortical hormone on the glycogenolytic action of epinephrine in skeletal muscle of the rat.

The differences in degree of the inhibitory influence of cortisone on the action of epinephrine in the several muscles appears to be correlated with the relative sensitivities of each muscle to the individual hormones when given alone. Thus, the relative sensitivity of the muscles to cortisone (to increase glycogen) is rectus femoris>abdominal>>>diaphragm. The relative sensitivity of the muscles to epinephrine is abdominal>rectus femoris=diaphragm. However, the inhibitory action of cortisone on the effects of epinephrine was greatest in the rectus femoris (complete inhibition), was intermediate in the abdominal muscles, and was not demonstrable in the diaphragm. Apparently a hormonal antagonism exists between epinephrine and cortisone which is particularly noticeable in the rectus femoris muscle. It appears that the two hormones act competitively on some mechanism concerned with glycogen deposition or break-down because increasing the dose of epinephrine over-rides the inhibitory effect of cortisone.

In the experiments which determined the optimum dose of epinephrine, it was noted that in the recovery phase, two to three hours after administering epinephrine, the glycogen level in the diaphragm was significantly higher than the initial level. This was not observed in the other muscles. To explain this, it is suggested that the increase in blood sugar, induced by epinephrine, was sufficient to stimulate an increased output of insulin, which in turn favored the deposition of excess amounts of glycogen in the diaphragm. Illingworth and Russell (1951) have shown that the diaphragm is exceedingly sensitive to insulin, and will respond to doses of this hormone, with increased glycogen deposition, under conditions which elicit no change in the gastrocnemius or heart muscles.

The importance of designating the specific skeletal muscle in studies concerned with the hormonal control of glycogen deposition has recently been stressed (Leonard, 1953). The results of the experiments reported here extend and emphasize this observation. It is also imperative in studying muscle glycogen to maintain a uniform length of time that the animals are anesthetized before removing the muscles for assay. The glycogen levels of the muscles of normal rats which were anesthetized for one hour and 10 minutes were from 20 to 28% higher than in those animals anesthetized for only 10 minutes.

A possible explanation for the inhibition of epinephrine by cortisone observed in these experiments may be found in the recent reports that cortisone decreases the phosphorylase *a* concentration of muscle and thus inhibits the breakdown of glycogen (Kerpolla, 1952). Epinephrine, on the other hand, increases the concentration of this enzyme and thus promotes glycogenolysis (Sutherland and Cori, 1951; Kerpolla, 1952). It would seem that the regulation of the phosphorylase *a* content of the muscle might

represent the mechanism of action of the two competing hormones, epinephrine and cortisone. Such an explanation implies that testosterone has a different site of action when increasing muscle glycogen.

SUMMARY

Comparisons were made of the glycogenolytic effect of epinephrine in different skeletal muscles in normal female rats, and those which had been previously treated with testosterone propionate (1 mg. a day for three days) and cortisone acetate (2 mg. a day for three days), to elevate the glycogen levels. Epinephrine was given intraperitoneally, 5 μg. per 100 grams body weight, one hour before autopsy. The rectus femoris, abdominal and diaphragm muscles were employed.

It was found that testosterone did not influence the glycogenolytic effects of epinephrine in any of the muscles. In contrast, cortisone inhibited the glycogenolytic action of epinephrine, completely in the rectus femoris muscle, partially in the abdominal muscle, and not at all in the diaphragm. By increasing the dose of epinephrine 4 fold, however, it was possible to override the complete inhibitory effect of cortisone in the rectus femoris muscle.

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THE CONTENT OF PROTEIN NITROGEN AND OF NUCLEIC ACIDS IN THE EPITHELIUM OF THE ECTOCERVIX OF WOMEN*

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MOST of our information about the cyclic changes in the epithelial cells of the female reproductive tract deals with modifications of morphology. The chemical analysis of these cells has been limited mainly to histochemical observations on the content of iron, phosphatase, and the degree of basophilia (Wislocki, 1945) and the content of glycogen in the endometrium and in the epithelium of the cervix and vagina (Papanicolaou 1948).

In order to obtain information about quantitative changes in the chemical constituents of the epithelium of the ectocervix we have attempted to follow the changes in protein nitrogen, ribosenucleic acid (RNA) and desoxyribosenucleic acid (DNA) in this tissue during the phases of the menstrual cycle. The data obtained in these analyses are reported in this paper.

MATERIALS AND METHODS

Preparation of tissue: In the present investigation only the epithelium of the human ectocervix was used. The greater number of samples came from specimens obtained through hysterectomies, but a few samples were taken from biopsies. Only epithelium which appeared to be perfectly normal without erosions or signs of irritation or inflammation was used. The epithelium of the ectocervix could be easily separated from the stroma by scraping. The collected epithelium was either used for analysis immediately or it was suspended in a large volume of 50% alcohol and stored in the refrigerator.

Chemical Analysis: The values for protein nitrogen in Table 1 were obtained from aliquots of the original homogenate. About 5% of these values can be accounted for as nucleic acid nitrogen. Determinations on samples which were extracted for determination of nucleic acids gave values for protein nitrogen which were 10–20% lower, presumably due to the extraction of some proteins in addition to nucleic acid. Determination of protein nitrogen was carried out in part by adaptation of a procedure described by E. J. Boell (1945).

Unexpected methodological difficulties led to a considerable amount of preliminary work on the procedure for the determination of nucleic acids. Attempting to use the

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TABLE 1. CONTENT OF DESOXYRIBOSENUCLEIC ACID, RIBOSENUCLEIC ACID AND PROTEIN NITROGEN IN CERVIX EPITHELIUM DURING NORMAL MENSTRUAL CYCLE AND POST PARTUM

Day of cycle	DNA	RNA	PN	No. cells $\times 10^6$	RNA/cell mg. $\times 10^6$	PN/cell mg. $\times 10^6$
	μg.	μg.	μg.			
1	54.8	96.5		6.44	15.0	
2	46.1	33.8	291	5.42	6.25	53.7
3	17.1	21.4	110	2.01	10.6	54.7
	20.9	26.2	102	2.46	10.6	41.5
4	76.2	116.	601	8.96	12.9	67.0
	35.9	54.6	221	4.22	12.9	52.4
5	28.2	47.7	240	3.32	14.3	72.4
7	57.9	103.	638	6.82	15.1	93.6
	81.6	122.	527	9.61	12.2	54.8
10	57.9	104.	679	6.81	15.3	99.5
11	64.0	91.6	585	7.53	12.2	77.8
12	66.5	119.	771	7.82	15.2	98.6
	49.5	82.5	560	5.82	14.2	96.1
	91.3	155.	1010	10.7	14.5	94.2
	76.2	124.	674	8.96	13.8	75.4
	46.6	43.9	258	5.48	8.01	47.0
	70.6	98.5	595	8.31	11.9	71.7
13	19.8	32.2		2.33	13.8	
	89.0	124.	561	10.5	11.8	53.5
14	51.9	73.5	347	6.11	12.0	56.9
	75.1	96.7	721	8.83	10.9	81.8
	77.4	98.9	524	9.10	10.9	57.6
15	55.4	89.4	429	6.53	13.6	65.6
16	34.2	44.6	271	4.03	11.1	67.5
	58.9	88.6	585	6.94	12.7	84.2
	64.8	93.8	449	7.62	12.3	59.0
18	67.5	98.9	514	7.94	12.4	64.8
19	37.9	67.1	280	4.46	15.0	62.8
20	91.5	96.0	464	10.8	8.90	43.0
	47.7	66.0	412	5.62	11.80	73.1
	75.9	108.	439	8.92	12.1	49.3
22	48.4	66.0	298	5.70	11.6	52.3
24	68.0	102.	270	8.00	12.7	46.3
26	143.	187.	1010	16.8	11.1	60.1
28	123.	235.	1092	14.5	16.2	75.2
30	69.3	67.5	445	8.15	8.30	54.6
34	57.6	77.7	394	6.78	11.4	58.1
Post partum	48.1	81.5	312	5.65	14.4	55.3
	25.8	36.8	170	3.04	12.1	55.7
	29.6	35.3	239	3.49	10.1	68.5
	96.5	134.	432	11.3	11.9	38.2
	115.	156.	557	13.6	11.4	41.0
	89.0	124.	415	10.5	11.8	39.5
	36.0	43.3	202	4.23	10.2	47.7
	30.1	43.9	186	3.55	12.4	52.3

colorimetric determination (Schneider, 1945) as modified by Euler and Hahn (1946), we noticed in extracts from many samples a color strikingly different from the color in standard solutions. In the phloroglucinol tests for RNA we observed colors from amber to dark brown and in the diphenylamine reaction for DNA a purple color instead of the green and blue of the respective standards. Correspondingly absorption curves were obtained with a peak at 460 to 470 mμ for the RNA reaction and at 540 mμ for the DNA reaction instead of the maxima at 620 and 600 mμ respectively for the standard solutions.

The changes in the absorption curves were also observed in extracts obtained with 1 N NaOH, according to Schmidt and Thannhauser (1945), and in the perchloric acid

extracts used in the method of Ogur and Rosen (1950). As a result of these preliminary tests we adopted the method of Ogur and Rosen for the extraction of NA in cervix samples, determining the nucleic acid content in the extracts as nucleic acid phosphorus.

In using the perchloric acid extraction the time which is required to remove RNA varies for different tissues and has to be determined separately in each instance. Tests on cervix samples with low mucin contents in which a colorimetric check was possible, indicated that an extraction time of 29 hours removed all RNA without removing any DNA.

RESULTS

The values obtained in the determinations of protein nitrogen, RNA, and DNA in cervix epithelium are compiled in Table 1. In the second to fourth column are listed the raw data for individual samples. In order to make such figures comparable they are usually calculated per unit of dry weight or wet weight. However, in analyzing the cervix epithelium during the various phases of the menstrual cycle the main interest lies, obviously, in the changes occurring in the epithelial cells in response to humoral and nutritional factors. Therefore the epithelial cell is regarded as the significant biological unit for which our data have to be calculated in order to be meaningful from the point of view of cellular physiology. This requires that the data obtained in our analysis be expressed per cell.

Until recently the expression of chemical data per cell seemed impracticable because of the difficulty of estimating the cell number in individual samples of tissue. Recent investigations indicate that the DNA content per cell is constant for all the tissues of any one species (Mirsky and Ris, 1951). The DNA content of the tissues of the human body is known to be close to 8.5 μg. $\times 10^{-7}$ per cell (Davidson et al., 1951). By dividing the DNA content of the samples by this figure the number of cells in the respective sample can be calculated. From such calculations we obtained the values for the cell numbers in our samples which are listed in column 5 of Table 1, and the corresponding values for PN and RNA per cell are given in columns 6 and 7 of the same table. It can be seen that the figures for the same day of the period scatter considerably. However, an apparent accumulation of high values for the PN and to a lesser extent for the RNA can be observed for the period from the 7th–12th day. Pooling the data for periods from the 5th–18th day and 19th–4th day, the number of samples became sufficiently large to give statistically significant differences for the PN (Table 2). The higher value was found for the first half of the menstrual cycle. These results seem to indicate that the PN content per cell in the cells of the cervix epithelium is higher during the first half of the menstrual cycle and that the maximum values are probably found in the period from the 8th to the 12th day of the cycle. Although our data show slightly higher values for RNA from the 5th–18th day, this apparent elevation proved not to be statistically significant.

TABLE 2. CONTENT OF RIBOSENUCLEIC ACID AND PROTEIN NITROGEN PER CELL OF CERVIX EPITHELIUM. MEANS AND STATISTICAL SIGNIFICANCE CALCULATED FROM DATA POOLED FOR 14 DAY PERIODS

	Days	Mid Point	No. Observ.	$\overline{X}$ Mean	$S\overline{X}$ Stand. dev.	Critical ratio*
RNA	5–18	11.5	20	12.7	1.8	.67
	19– 4	25.5	13	11.7	2.5	$P = .50$
PN	5–18	11.5	20	73.6	16.6	3.6
	19– 4	25.5	13	56.2	10.0	P = less than .01

* These figures are calculated according to the formula:

$$\frac{\overline{X}_1 - \overline{X}_2}{\sqrt{\frac{S\overline{X}_1^2}{n_1 - 1} + \frac{S\overline{X}_2^2}{n_2 - 1}}}$$

The values for RNA and PN in several samples obtained post partum were found to be low (Table 1), corresponding to the figures obtained during the second half of the menstrual cycle.

DISCUSSION

It is known from morphological observations that the cervical cells of different individuals vary greatly in the timing and the extent of their response to the factors which bring about the changes during the menstrual cycle (Wollner, 1937). Therefore it was to be expected that our data would show a considerable scattering and that only large numbers of samples would indicate statistically significant differences. The results obtained so far show that the protein content of the cervical epithelium is elevated during the period from the 5th to 18th day of the mentrual cycle. A maximum increase seems to be reached between the 7th and 12th day. This time coincides with the advanced follicular phase (Papanicolaou, 1948), and with the increase in the levels of estrogen and gonadotropic hormone. It is also a period during which the height of the cervical epithelium increases considerably. In the earlier part of the follicular phase mitotic activity is supposed to contribute the greater part of this increase; in the later part increase in cell size plays the dominant role (Papanicolaou, page 34, 1948). It would seem, then, that cell enlargement and protein accumulation coincide and that the increase in protein content is a response of the anabolic processes of the cervix cell to stimulation by estrogen. Following this argument the low values of PN in the post partum samples could be explained by the sharp drop of the estrogen level during the time of parturition.

During the luteal phase we observed a decrease in the protein content of the cervical cells. During this phase a change in the appearance of the cervical cells occurs which has been described as a loss of turgescence and as a process of involution of the cells of the superficial layers of the cervix epithelium (Papanicolaou, page 35, 1948). Estrogen as well as progester-

one are present in large quantities at this time and the presence of the latter must be responsible for the morphological transformation of the epithelium and the concomitant changes in the protein level.

The interpretation of our results seems to be corroborated by the observations of a dependence of protein formation in cells of other tissues of the female reproductive tract upon hormonal activity. Csapo (1950) found that the accumulation of the protein actomyosin in the myometrium is greatly enhanced by estrogen, and Mueller (1952) could show with tracer techniques that this accumulation is due to an accelerated rate of protein synthesis. Alfert and Bern (1951) demonstrated that the nuclear volume in the cells of uterine glands doubles after injection of estrogen into castrated animals. This increase in nuclear volume, too, was accompanied by a proportional increase in the protein content of the nucleus.

SUMMARY

1. Determinations of the content of protein nitrogen, ribosenucleic acid and desoxyribosenucleic acid in human cervix epithelium have been carried out.

2. A significant elevation of the protein content per cell of the cervix epithelium has been found for the first half of the menstrual cycle with a probably maximum in the period from the 7th to 12th day. The RNA content per cell was found not to change significantly during the menstrual cycle.

Acknowledgment

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THE PRODUCTION OF HYPERTENSION, NEPHROSCLEROSIS AND CARDIAC LESIONS BY METHYLANDROSTENEDIOL TREATMENT IN THE RAT[1]

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THE "hyalinosis syndrome" is the name applied, by Selye (1946, 1950), to the cardiovascular-renal changes which occur in salt-treated, unilaterally nephrectomized rats administered such steroids as desoxycorticosterone acetate (DCA) or Reichstein's Compound S (desoxocortisone) for prolonged periods. The characteristic changes of this syndrome are hypertension, renal and cardiac hypertrophy, nephrosclerosis, glomerular hyalinization, "rheumatic-like" cardiac nodules and frequently periarteritis nodosa. Up to the present time such changes have been observed only after treatment with steroids known to possess appreciable mineralocorticoid activity. This fact, plus the observation that certain stresses, as well as the administration of lyophilized anterior pituitary powder (LAP) or alkaline pituitary extract (APE) also produced the hyalinosis syndrome, led Selye to postulate (1946, 1950) that similar pathological conditions in the human might be pathogenetically related to hyperfunction of the anterior pituitary-adrenocortical system involving the increased secretion of mineralo-corticoids.

Selye and his co-workers have further observed that experimental nephrosclerosis and hypertension were more readily produced in female or castrate male rats than in intact males (Selye, Hall and Rowley, 1943; Selye and Pentz, 1943; Selye, Sylvester, Hall and Leblond, 1944) and that methyl-testosterone antagonized the nephrosclerotic effect of DCA administration (Selye and Rowley, 1944). These observations were believed to depend upon the protective effect of androgens on renal tissue as a result of their inherent renotropic activity (Selye, 1939, 1940, 1941; Selye and Friedman, 1941). However, the high androgenic potency of methyl-tes-

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tosterone precluded the clinical application of such an experimental observation to the treatment of hypertension. The ideal compound for clinical purposes, would, of course, be one with high renotropic and comparatively no androgenic activity. On the basis of recent reports (Gordan, Eisenberg, Moon and Sakamoto, 1951; Homburger, Kasdon and Fishman, 1950), methylandrostenediol (MAD)[3] appeared to closely conform to these requirements. Consequently, the following experiments were performed to determine whether MAD possessed an inhibitory action similar to that of methyl-testosterone on the changes induced by DCA administration in the rat. It was felt to be of considerable interest, therefore, when it was found that MAD was capable not only of potentiating the effects of DCA but also of producing similar changes when administered alone.

MATERIALS AND METHODS

Experiment 1.—Thirty-two female, hooded rats, averaging 134 gm. in weight, were divided into 4 equal groups and treated in the following manner. Group I served as controls. The rats in Group II received DCA in daily subcutaneous injections of 0.5 mg. contained as a microcrystalline suspension in 0.1 ml. of distilled water. The rats of group III were similarly treated with 2.5 mg. of MAD per day, and those of group IV received combined DCA and MAD treatment in the same manner as outlined for groups II and III. The experiment was terminated after 7 weeks.

Experiment 2.—Three groups, each of 10 female, hooded rats, weighing 83–100 gm. were treated as follows: Group I was untreated and acted as controls. The rats of groups II and III received daily subcutaneous injections of 10 mg. of MAD and 2 mg. of DCA respectively, both steroids being administered as a microcrystalline suspension in 0.2 ml. of distilled water. The duration of the experiment was six weeks.

All rats were unilaterally nephrectomized and given 1% NaCl to drink *ad libitum* throughout each experiment. The diet consisted of a balanced synthetic ration known to be adequate for the maintenance of this strain of rat over long periods (Skelton, 1950). The body weights were determined at the beginning and at the end of each experiment and the saline consumption of each rat was measured daily. Systolic blood pressure determinations were made by the tail plethysmographic method (Williams, Harrison and Grollman, 1939) with the rats under light ether anesthesia. At the conclusion of each experiment, the rats were killed by exsanguination and the heart, kidneys, adrenals, and thymus were promptly removed. After fixation in Bouin's solution for at least 48 hours, they were dissected free of fat, accurately weighed on a chainomatic balance, sectioned at 6 u and stained with haematoxylin and eosin for microscopic examination.

RESULTS

Experiment 1.—During the course of this experiment a mechanical difficulty partially prevented the rats from having free access to the diet at all times, a fact which may explain their slightly subnormal growth (Table 1). It should also be noted that during the experiment 1 rat died in group III

[3] (17α-methyl-Δ^5-androstene-3α-17α-diol).

and 2 in group IV. The results of the blood pressure determinations, and the measurements of saline intake are graphically presented in Figure 1. It can be seen that the saline intake of the DCA treated rats increased only slightly above that of the controls, while no such increase occurred in the rats receiving MAD. The saline consumption of group IV (DCA and MAD) increased to a level slightly greater than that observed in the groups receiving either steroid alone. Furthermore, no elevation of the blood pressure was detected in the DCA treated rats while a significant ($P < 0.05$) hypertension occurred in those receiving MAD, despite the absence of any increase in their 1% NaCl intake. The blood pressure of the rats in group IV was very significantly ($P < 0.01$) greater than that of either the control or DCA treated animals.

TABLE 1. THE BODY AND ORGAN WEIGHTS OF SALT-TREATED UNILATERALLY NEPHRECTOMIZED FEMALE RATS TREATED FOR 7 WEEKS WITH SMALL DOSES OF DCA AND MAD ALONE AND IN COMBINATION

Group and treatment	No. of rats	Body weight—gm.		Organ weights in mg/100 gm. body weight			
		Iritial	Final	Kidney	Heart	Thymus	Adrenals
I Control	8	131 ± 4	146 ± 5	531 ± 10	399 ± 12	88.9 ± 8.7	22.3 ± 2.1
II DCA	8	135 ± 4	150 ± 3	649 ± 26	432 ± 13	107 ± 2.2	20 ± 2
III MAD	7	135 ± 3	155 ± 6	802 ± 26	465 ± 15	45.5 ± 4.9	24 ± 1.1
IV DCA MAD	6	136 ± 1	155 ± 9	925 ± 72	580 ± 17	47.8 ± 5	24.2 ± 1.7

All weights accompanied by the standard error of the mean.

The organ weights presented in Table 1 demonstrate a striking parallel between the renal and cardiac weights and the blood pressure. Thus, significant renal hypertrophy was present in the rats treated with either DCA or MAD (groups II and III), the hypertrophy in the latter group being significantly greater ($P < 0.01$) than in the former. The combined DCA and MAD treatment of group IV produced the greatest renal enlargement that was observed in this experiment. Consistent with the absence of hypertension, was the absence of cardiac hypertrophy in the rats treated with DCA alone, while significant cardiac enlargement was found in the animals receiving either MAD alone or MAD and DCA in which hypertension was present. Marked thymic involution occurred in the rats of both groups III and IV where MAD was administered either alone or in combination with DCA.

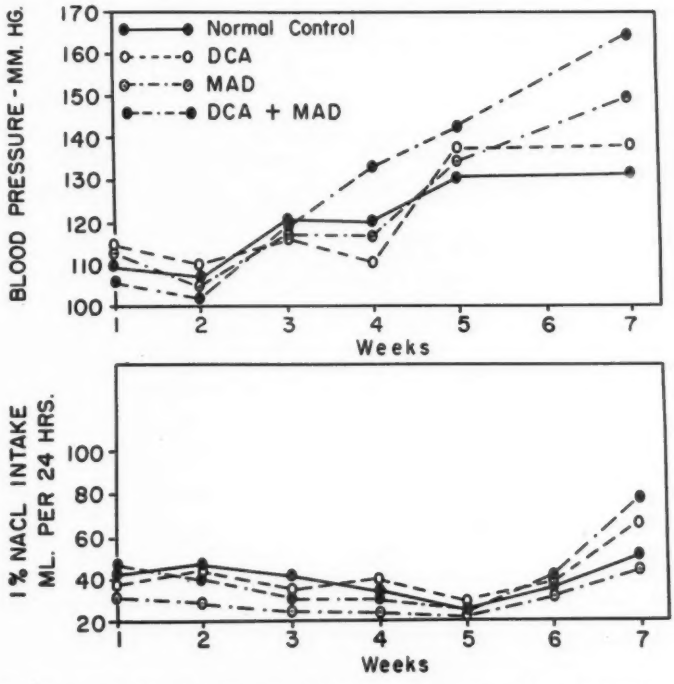


FIG. 1. Changes in blood pressure and saline consumption of the rats in experiment 1. The average systolic blood pressure of each group is expressed in mm. of mercury as the mean of 3 weekly determinations. The saline consumption was measured in ml. and is expressed for each group as the average 24-hour intake per rat per week.

Microscopic examination of the kidneys showed only minimal hyaline glomerular and arteriolar changes in the rats treated with DCA or MAD alone (groups II and III), while there was definite hyalinization of glomerular capillary tufts and arterioles in the rats receiving the combined steroid treatment (Group IV). Similarly, the hearts of the animals treated with DCA or MAD separately (groups II and III respectively) showed only slight, scattered areas of myocardial necrosis and cellular infiltration, which was increased in severity and incidence in the rats of group IV treated with both DCA and MAD. Lesions similar to periarteritis nodosa were detected in the pancreatic and mesenteric arterioles of the latter group only, another evidence of the greater severity of the changes produced in this group.

Experiment 2.—The results of this experiment are presented in Figure 2 and Table 2 in a manner similar to experiment 1. During the experiment 2

rats died in group III and as Table 2 indicates the rats of both experimental groups weighed less than the controls when the experiment was terminated. The upper part of Figure 2 shows that a highly significant hypertension developed in the rats of both experimental groups. The final decline in the blood pressure of the DCA treated animals was probably the result of the progressively increasing severity of the steroid intoxication, which was also indicated by the presence of a simultaneous

TABLE 2. THE BODY AND ORGAN WEIGHTS AND QUANTITATIVE EVALUATION OF THE PRINCIPLE ORGAN CHANGES IN SALT-TREATED, UNILATERALLY-NEPHRECTOMIZED, FEMALE RATS TREATED FOR 6 WEEKS WITH LARGE DOSES OF DCA AND MAD

Group and treatment	No. of rats	Body weight—gm.		Nephrosclerosis		Cardiac lesions		Organ weights in mg/100 gm. body weight			
		Initial	Final	Per cent incidence	Per cent severity	Per cent incidence	Per cent severity	Kidney	Heart	Thymus	Adrenal
I Control	10	88±4	154±3	0	0	0	0	578±18	349±46	145±15	20.2±1.2
II MAD	10	96±5	147±5	100	64	90	36	1752±56	537±22	0	31.9±2.8
III DCA	8	96±3	140±6	100	60	88	43	1240±105	568±32	36.6±18.6	23.7±2.6

All weights accompanied by the standard error of the mean.

weight loss in these rats. As seen in the lower part of the figure the characteristically rapid onset of polydipsia in the DCA treated rats contrasts sharply with the gradual and less marked rise in the saline consumption of the animals receiving MAD.

The gross observations at autopsy were striking. There was marked renal hypertrophy in both experimental groups, but it was particularly pronounced in the rats treated with MAD. In this group the kidneys were large, pale and soft and showed numerous small, white and hemorrhagic spots scattered over the surface, (Figs. 3 and 5). The kidneys from group III were smaller and redder but showed similar white and red areas on the surface (Figs. 3 and 5). Examination of cross-sections of the kidneys showed that the hypertrophy in both groups was limited principally to the renal cortex. Of interest is the fact that approximately the same degree of cardiac hypertrophy was present in both MAD and DCA treated rats and that small grey-white areas were visible on their epicardial surfaces (Fig. 4) The severity of the renal and cardiac changes have been quantitated by grading the lesions both grossly and microscopically from 0 to 4 plus and then expressing the total number of pluses in any one group as a percentage of the theoretical maximum. The tabulated results (Table 2) indicate that the incidence and severity of both the nephrosclerosis and cardiac lesions were almost identical in the two experimental groups. As in the first experiment, the thymus of the MAD-treated rats was completely atrophic while in the animals receiving DCA there was irregular involution

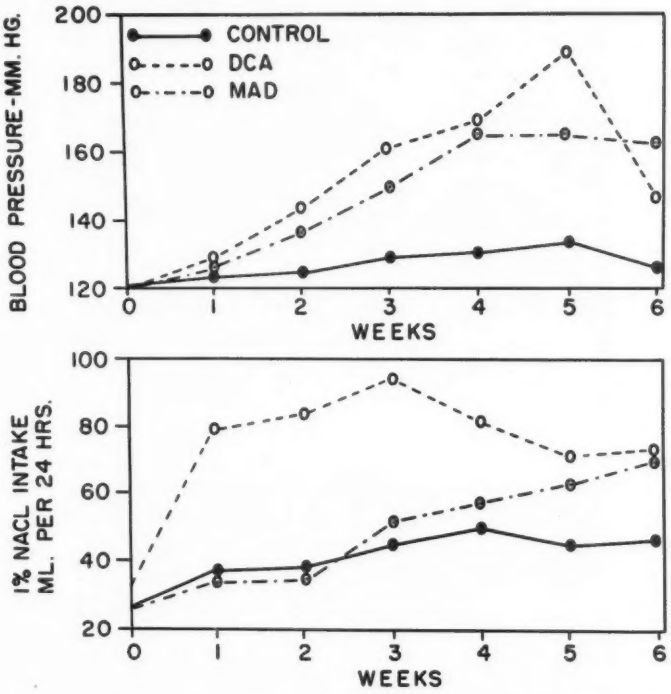
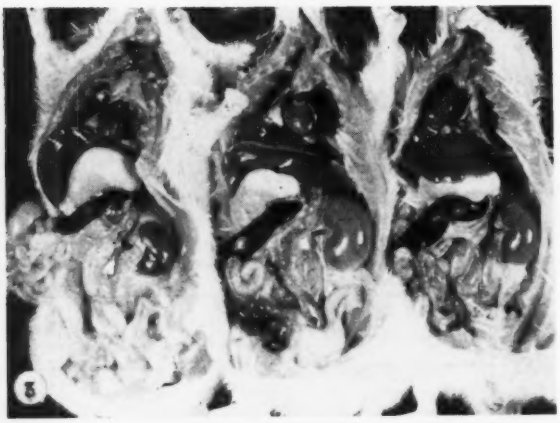


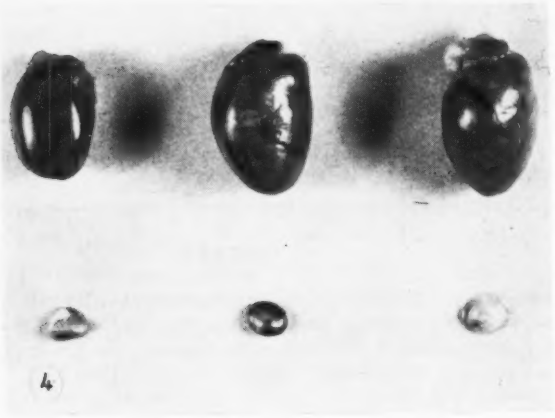
FIG. 2. Changes in blood pressure and saline consumption of the rats in experiment 2. The average systolic blood pressure of each group is expressed in mm. of mercury as the mean of 3 weekly determinations. The saline consumption was measured in ml. and is expressed for each group as the average 24-hour intake per rat per week.

(Fig. 3). No gross changes were seen in the adrenals of the DCA-treated rats, but in the animals administered MAD these glands were uniformly enlarged and showed scattered, red, hemorrhagic areas on the surface (Fig. 4).

The weights of the kidney, heart, thymus and adrenals from these rats are presented in Table 2 and they essentially confirm the gross observations listed above.

Microscopic examination showed that the renal and cardiac lesions produced by MAD and DCA administration under the present experimental conditions were similar. The renal changes were characterized by hyaline degeneration of the small arterioles, glomerular enlargement and hyalinization, tubular dilatation, and the hyaline cast formation (Figs. 6 and 7). The lesions in the hearts varied from focal myocardial necrosis with minimal fibroblastic proliferation to large, granulomatous areas with hyaline





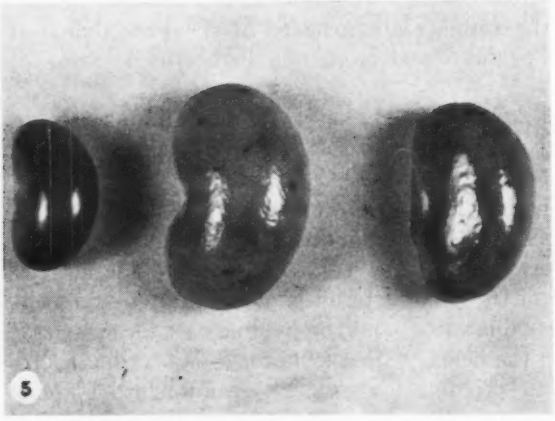


PLATE I

infiltration of the myocardium (Fig. 8). Frequently the arterioles also showed hyaline material in the sub-intima, media or adventitia with an increased number of Anitchkow or "caterpillar" cells in the perivascular region (Figs. 9 and 10). Periarteritis of the mesenteric and pancreatic vessels was not prominent in either experimental groups but this lesion occasionally occurred. Figure 11 illustrates such a periarteritic lesion of a pancreatic mesenteric artery in a rat treated with MAD.

The adrenals from the DCA treated rats (Group II) did not differ from the adrenals of the normal controls. However, in the MAD treated animals, the adrenals showed uniform thickening of the fibrous capsule and frequent periarteritic changes of the periadrenal arterioles. The cortex showed numerous areas of almost complete necrosis and hemorrhage which probably accounts for the mottled red appearance of the glands grossly (Fig. 12). The adrenocortical cells of the reticularis and fasciculata frequently contained a single large vacuole making them resemble fat cells while adjacent to the necrotic areas and frequently beneath hyalinized surface arterioles, dense, eosinophilic, hyaline-like material infiltrated between the cell cords (Fig. 13). Microscopic examination of such areas under oil immersion revealed a high incidence of single or multiple hyaline or colloid inclusion bodies in the cytoplasm of large, multi-vacuolated adrenocortical cells (Fig. 14).

DISCUSSION

The first experiment was designed to determine whether MAD inhibited the over-dosage effects of DCA in a manner similar to methyl-testosterone However, the results indicated that this androgenic compound in itself induced hypertension and cardiac hypertrophy and aggravated the effects of DCA when administered with it. Because of the small doses employed, only minimal lesions were produced by either DCA or MAD alone. The slight renal hypertrophy which was produced by these steroids may have been a manifestation of their renotrophic rather than their nephrosclerotic

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FIG. 3.—Three rats of approximately equal weight are from left to right: Control, MAD treated and DCA treated. Note the renal and cardiac hypertrophy of the treated rats, the dark spots on the surface of the kidneys contrasting with their pale color, and the complete thymic atrophy in the MAD treated animal.

FIG. 4. The hearts and adrenals of the above rats showing the great cardiac enlargement and the white areas on the epicardial surface of the hearts of the treated rats. Note the hemorrhagic appearance of the adrenal from the MAD treated animal.

FIG. 5. The kidneys from these same rats clearly showing the enlargement and the irregular surface of the kidneys from the experimental animals. The kidney from the MAD treated rat is noticeably larger than that from the DCA treated animal and the dark spots are plainly visible.

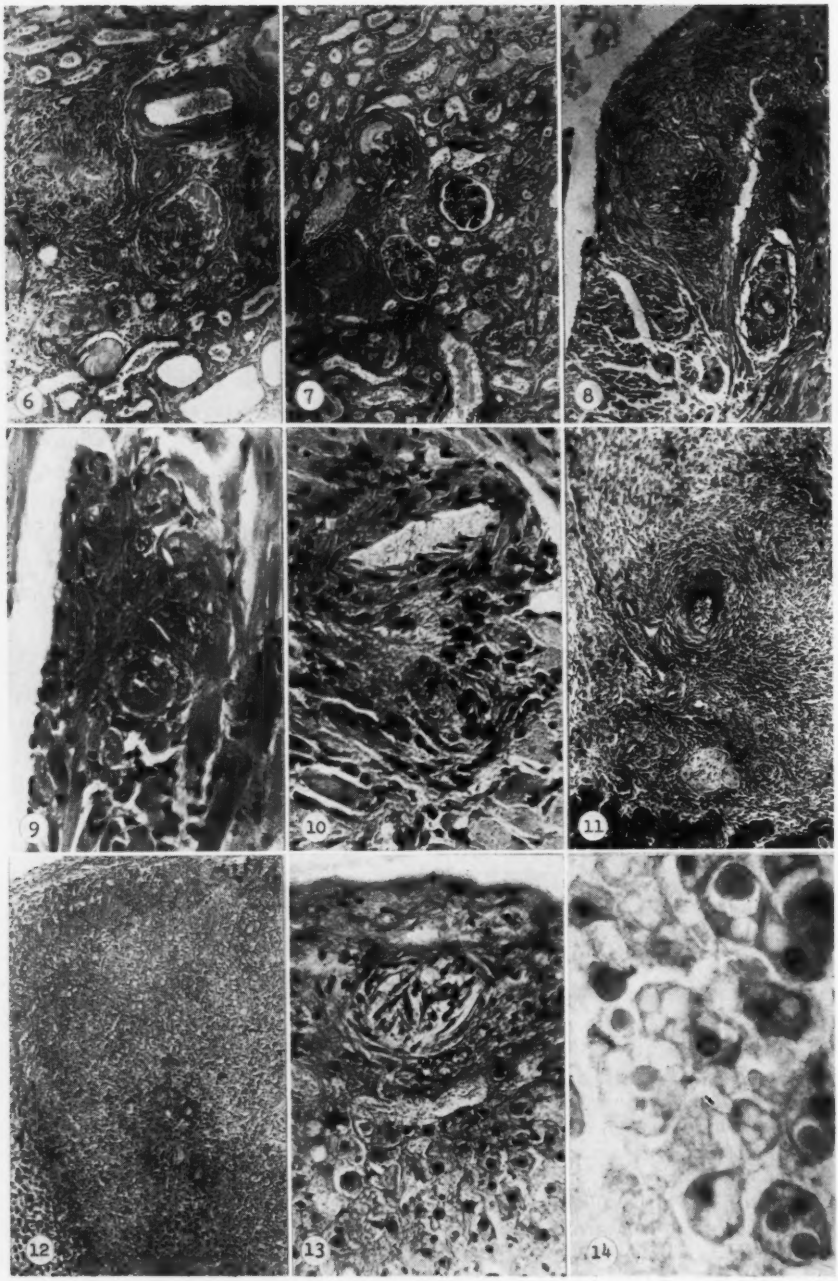

PLATE II

action, the result of fluid retention or perhaps both. The failure of cardiac hypertrophy to develop in the DCA-treated rats was not surprising in the absence of hypertension, although minimal microscopic lesions of the heart were detected. On the other hand, the cardiac enlargement and more severe microscopic lesions observed in the MAD-treated rats are consistent with the significant elevation in their blood pressure. Thus, as the effects of DCA and MAD alone were essentially similar, the greater severity of all changes in the group receiving both steroids is readily understandable. It is difficult to say whether this is a true potentiation or simply an additive effect although the latter seems more likely.

It was apparent from the first experiment that the doses of both DCA and MAD were inadequate to produce consistent or pronounced changes in either the blood pressure of the rats or in the weights and histologic structure of the hearts or kidneys. Hence the dose level of each steroid was increased four-fold in the second experiment. As previously seen the results of this experiment have adequately demonstrated the close similarity between the effects of DCA and MAD overdosage under these ex-

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FIG. 6. Kidney from a rat treated with MAD showing the glomerular hyalinization, arteriolosclerosis, tubular dilatation and hyaline casts. One glomerulus has been completely destroyed while another is only partially destroyed with fibrinoid material in the glomerular space. Hematoxylin and eosin; ×100.

FIG. 7. Kidney from a rat treated with DCA showing somewhat less severe glomerular hyalinization, arteriolosclerosis of an afferent arteriole, tubular dilatation and hyaline casts. Hematoxylin and eosin; ×100.

FIG. 8. Cardiac muscle from a rat treated with MAD showing the deposition of hyaline material, the surrounding fibroblastic proliferation, and the thickening of arteriolar walls. Hematoxylin and eosin; ×100.

FIG. 9. Photomicrograph showing an area containing hyaline material about a small arteriole whose wall is thickened by the subintimal deposition of similar material. Anitschkow myocytes are present in this small lesion. Hematoxylin and eosin; ×250.

FIG. 10. Photomicrograph of heart of a DCA treated rat showing a similar but less severe myocardial lesion than that shown in the previous figure. Hematoxylin and eosin; ×275.

FIG. 11. A small pancreatic artery from a rat treated with MAD showing the subintimal deposition of hyaline material and the perivascular accumulation of inflammatory cells. Hematoxylin and eosin; ×100.

FIG. 12. A low power photomicrograph of the adrenal from a rat treated with MAD showing the extensive cortical necrosis. Hematoxylin and eosin; ×100.

FIG. 13. Another region of the same adrenal cortex as in Figure 12 showing early vascular changes and the surrounding deposition of hyaline material. Hematoxylin and eosin; ×250.

FIG. 14. Photomicrograph of the edge of the necrotic region shown in Figure 12. Note the greatly enlarged cortical cells containing large vacuoles and colloid or hyaline inclusion bodies in the cytoplasm of several of the cells. Hematoxylin and eosin; ×500.

perimental conditions. It is noteworthy, however, that the changes elicited by these steroids are not identical. This is well shown by the rapid onset of polydipsia in the rats receiving DCA in contrast to the slow increase in saline consumption of the MAD treated animals. In this respect the effect of MAD treatment resembles that of LAP (lyophilized anterior pituitary) administration more closely than DCA. Furthermore, LAP characteristically produces large, pale kidneys similar to those observed in the MAD treated rats of the present experiment, while DCA produces kidneys which are neither as large nor as pale.

The effects of these two steroids differed in other ways. Whereas DCA caused little gross or microscopic change in the adrenal glands, MAD produced significant enlargement, fibrous thickening of the capsule and fatty changes of the cortical cells similar to those produced by methyl-testosterone (Selye, 1940; Selye, Rowley and Hall, 1943) frequent adrenal periarteritis, focal cortical necrosis with hyaline deposition in the cortex and hyaline or colloid inclusion bodies within the cortical cells. Similar inclusion bodies have been described by Selye and Stone (1950) in the adrenals of rats treated with LAP alone, LAP and DCA, LAP and methyl-testosterone, LAP, methyl-testosterone and thyroxine or LAP, DCA and thyroxine. These authors interpreted this phenomenon as a result of storage of some cortical hormone principle under conditions where "the stimulus for hormone production is greater than that for granule discharge." The finding of similar inclusion bodies in the adrenocortical cells of rats treated with MAD alone makes this interpretation less attractive since there was no apparent stimulus for increased functional activity of the adrenal under the present conditions. The concomitant findings of adrenal periarteritis, adrenal-cortical necrosis, and hyaline material in the sinusoids suggests that the formation of hyaline or colloid inclusion bodies may be a degenerative phenomenon. At any rate, the observation is an interesting one and certainly warrants further study.

The present observations represent the first time that an androgenic steroid has been shown to produce hypertension and other cardiovascular-renal changes characteristic of the "hyalinosis syndrome." Grollman, Harrison, and Williams, (1940) have observed hypertension in rats treated with a variety of steroids including testosterone propionate, but they did not include histological studies in their experiments. These authors concluded, that the hypertension was probably secondary to renal changes induced by the steroids rather than to the retention of salt and water directly. Since that time the importance of sodium in the pathogenesis of experimental hormonal hypertension and the accompanying cardiovascular-renal changes has been repeatedly demonstrated (Selye, 1943; Selye and Stone, 1943; Selye and Pentz, 1943; Selye, Minzberg and Rowley, 1945;

Braun-Menendez and Prado, 1950; Braun-Menendez, 1951). Whether the sodium ion is of equal importance in the pathogenesis of the hypertension, and "hyalinosis" changes induced by MAD administration is not established by the present experiments. However, it is noteworthy that hypertension occurred in the MAD treated rats of the first experiment in the absence of an increased NaCl intake and in the second experiment the saline consumption of the rats receiving MAD increased so slowly that a significant degree of hypertension was present at a time when the saline intake was little more than that of the control animals. This is in sharp contrast to the marked early increase in the NaCl consumption of the DCA treated rats which is then followed by a rise in the blood pressure.

Despite these observations, one cannot discount the role of sodium retention in the pathogenesis of the changes induced by MAD, since Kenyon, Knowlton, Sandiford, Koch and Lotwin, (1940) have likened the action of androgens on electrolytes to an adrenocortical effect. Recently metabolic experiments in the human have shown that MAD causes sodium retention along with the retention of nitrogen and potassium (Weissberg, Chevalley, Stern and McGavack, 1951). Furthermore, Fourman (1952) has shown that methyl-testosterone in man in doses of 100 mg. per day produces a retention of nitrogen, potassium and sodium and has presented evidence that the sodium, unlike that retained by DCA, remains extracellular. Perhaps such a selective extracellular sodium retention with increased extracellular fluid volume could explain the production of hypertension by MAD in the absence of a significantly increased NaCl intake. It is the opinion of the author that this aspect of the problem warrants careful consideration in future studies as a natural extension of the present observations.

Some of the findings in these experiments are relevant to the question of whether arteriolar nephrosclerosis is the cause or the result of vascular hypertension. It will be remembered that hypertension occurred in the MAD treated rats of the first experiment in the absence of recognizable renal and cardiac lesions, and even in the group receiving combined MAD and DCA treatment the pathological changes in these organs were not severe despite the presence of a considerable but early hypertension. Thus it would appear that in the sequence of events hypertension occurred first and was followed by the development of pathologic cardiovascular-renal changes.

What is the significance of the present observations? Certainly they show that antagonism to certain functional and anatomical effects of DCA is not a universal property of all androgens. Furthermore, they indicate that an androgenic steroid, in this case MAD, can produce changes very similar to those induced by DCA. The concept of a pathogenetic relationship be-

tween adrenocortical function and human hypertension and nephrosclerosis has been based to a great extent on the production of such lesions by DCA in the experimental animal. It is felt that the production of similar lesions by an androgenic substance cases some doubt on the validity of such a concept and at the same time emphasizes the necessity of a thorough study of the steroid hormones and related factors in the production of hormonal hypertension. Only by such a detailed and exhaustive study can the true significance of the endocrine system in the genesis of experimental hypertension and cardiovascular-renal disease be established.

SUMMARY

The effects of chronic administration of methylandrostenediol (MAD) and desoxycorticosterone acetate (DCA) have been studied in the unilaterally nephrectomized, female rat allowed 1% sodium chloride to drink. When these steroids were given alone in doses of 2.5 mg. per day and 0.5 mg. per day respectively, slight hypertension developed only in the MAD treated animals. When both steroids were given the hypertension was more pronounced and definite anatomical changes were detected in the kidneys and heart. The administration of MAD and DCA in daily doses of 10 mg. and 2 mg. respectively for a period of 6 weeks produced a progressive increase in the saline intake and a rise in the blood pressure of the treated animals. At autopsy the DCA treated rats showed renal and cardiac hypertrophy, marked arteriolar nephrosclerosis, glomerular hyalinization, tubular dilatation and cast formation, and cardiac lesions characterized by vascular and interstitial hyaline and granulomatous formation. The animals treated with MAD showed significantly greater renal hypertrophy, almost identical cardiac enlargement and similar but more marked renal and cardiac lesions. In addition MAD produced a peculiar adrenal cortical necrosis, characterized by hyaline material in the sinusoids and intracellular hyaline or colloid inclusion bodies. Marked thymic involution was characteristic of the rats treated with MAD. While the present experiments illustrate certain similarities in the changes produced by the chronic administration of these steroids to unilaterally nephrectomized, salt-treated rats, it is felt that there are sufficient variations to warrant the conclusion that fundamental differences exist in their mechanism of action.

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CHOLESTEROL BIOSYNTHESIS IN AN ADRENAL TUMOR[1]

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THE continuing implications of adrenal cholesterol in the biogenesis of the steroid hormones have led to considerable study of the biosynthesis of cholesterol in the adrenal gland. Adrenal tissue has been included in several studies of the conversion of C^{14}-acetate to cholesterol. Beef adrenal cortex slices (Srere, Chaikoff, and Dauben, 1948) and perfused swine adrenals (Schwenk and Werthessen, 1952) are capable of the transformation of acetate to cholesterol. Swine adrenal cortex slices are also known to yield 17 α-hydroxycorticosterone from acetate (Haines, Nielson, Drake, and Woods, 1951) and perfused beef adrenals afford 17-hydroxycorticosterone and corticosterone from acetate substrate (Zaffaroni, Hechter, and Pincus, 1951).

In that the normal adrenal is able to utilize sodium acetate-1-C^{14} as substrate in the synthesis of the steroid nucleus, interest in the synthetic processes of an adrenal tumor was considerable. An adrenal tumor[2] of human origin was studied using the isolated organ perfusion techniques previously described (Werthessen and Schwenk, 1952). During the course of the perfusion sodium acetate-1-C^{14} was injected, and with fresh portions of perfusion fluid the following were added: ACTH, cortisone, Δ^4-androstene-3,17-dione, progesterone, desoxycorticosterone, and Δ^5-androstene-3β,17β-diol. The results of these studies will be reported elsewhere. The ability of the adrenal tumor to synthesize cholesterol from acetate is described herein.

The necessity of rigorous purification of cholesterol isolated from organ perfusions has been noted (Schwenk and Werthessen, 1952). Thus several purification procedures were investigated using the sterol mixture isolated from the adrenal tumor tissue by use of digitonin.

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[1] The work described herein was supported in part by a grant-in-aid from the Texas Heart Association.

[2] Dr. A. Segaloff describes the tumor as an adrenal cortical tumor with marked anaplasia, but with no evidence of invasion of the capsule or extension beyond the adrenal.

EXPERIMENTAL

Melting points were determined on a Kofler block unless otherwise noted. Radioactivity assays were made from cholesterol samples directly plated onto aluminium disks from methyl alcohol solution. Assay samples weighed one to five milligrams and were confined to an area of two square centimeters. Self-absorption corrections were applied to all samples to a standard comparison thickness of one milligram per square centimeter. A TGC-2 thin window tube was used with a Tracerlab 100 Scaler for counting.

The tumor was removed from a 15 month old child; it was connected to the perfusion pump under sterile conditions via the major artery within 45 minutes after the circulation was cut. Perfusion was with human blood diluted with an equal volume of White's solution. Additions of ACTH, cortisone, Δ^4-androstene-3,17-dione, progesterone, desoxycorticosterone, and Δ^5-androstene-3β,17β-diol were made with fresh perfusion fluid, one cycle being allowed for each compound under investigation, at the end of which the blood-White's solution was removed. Fresh perfusion fluid was then recycled through the tumor until the next compound was injected. Sodium acetate-1-C^{14} was injected in nine portions throughout the perfusion, a total of 1.78 mC. being used.

At the conclusion of the perfusion period of twelve hours the adrenal was in a viable state as indicated by histological examination. A cut was made in the surface of the tumor opposite the artery supplying the perfusion fluid. Fresh White's solution then injected exuded from the cut face, permitting an estimate of at least 80% of the organ having received the perfusion fluid properly. The tumor weighed 109 g.

The tumor was hydrolyzed in 30% aqueous potassium hydroxide; the hydrolysate was extracted with pentane in a continuous extractor, and the residue so obtained treated with digitonin (Windaus, 1909). Regeneration with pyridine (Schoenheimer and Dam, 1933), followed by several washings of the ether extract with 10% hydrochloric acid, then water, yielded the crude sterol mixture.

Recrystallization of the pentane soluble portion from methyl alcohol yielded 200 mg. of impure cholesterol, melting at 127–133° (Capillary), with a specific activity of 1566 counts/min./mg. This material was subjected to the several purification procedures described.

Chromatography of the sterol fraction was conducted on silica gel. Benzene elutions sufficed to remove more than 90% of the adsorbed sample from the column. Paper chromatographic analysis of several fractions was performed using a β-phenoxyethyl alcohol-heptane system (Neher and Wettstein, 1952). The cholesterol was purified via the dibromide using the procedure of Schwenk and Werthessen.

RESULTS

Chromatography of 105 mg. of impure cholesterol afforded 96.2 mg. of still impure cholesterol, of specific activity 1251 counts/min./mg. Rechromatography of this fraction gave pure cholesterol of constant radioactivity in the middle fractions; however, earlier fractions and latter

TABLE 1. FINAL CHROMATOGRAPHY OF TUMOR CHOLESTEROL

Fraction	Weight, mg.	Melting point	Specific activity, counts/min./mg.
10	4.2		2335
11	9.3	144–146°	906
12	11.3	148–149°	738
13	9.6	144–146°	720
14	6.9	147–148°	1125
15	6.8		1387
16–19	9.4		1735

fractions showed much higher specific activity than the cholesterol isolated. The data are presented in Table 1. The data suggest that careful control and individual assay of each eluted fraction is necessary for a radiochemically pure sample. The increased specific activity of the fore and after fractions indicates the presence of other components (Fig. 1).

Fractions 11–13 were purified via the dibromide, and gave cholesterol melting at 149.0–149.5°, with a specific activity of 631 counts/min./mg. Recrystallizations from methyl alcohol did not change the melting point which was not depressed on admixture with an authentic cholesterol sample. The specific activity of the recrystallized cholesterol was also not altered. Infrared analysis of this fraction indicated identity and spectroscopic purity.[3] Co-chromatography of the sample on paper with authentic cholesterol indicated identical behavior as regards migration and color test with the Liebermann-Burchard reagent.

Direct conversion of a portion of the impure sterol mixture to the dibromide (Schwenk and Werthessen, 1952) and regeneration of the precipitated dibromide with zinc-acetic acid and recrystallization gave a fraction, melting 145–146° with a specific activity of 705 counts/min./mg. Chromatography of this material and rechromatography of the main fractions melting 148–150° yielded a series of fractions assayed separately. A constant specific activity was found for these cholesterol fractions, as indicated by the plot of the data in Figure 1. The higher counting materials have been eliminated by the bromination-debromination procedure as indicated by comparison of the two curves on Figure 1.

[3] The infrared analysis was kindly performed by Dr. H. Rosenkrantz, Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts.

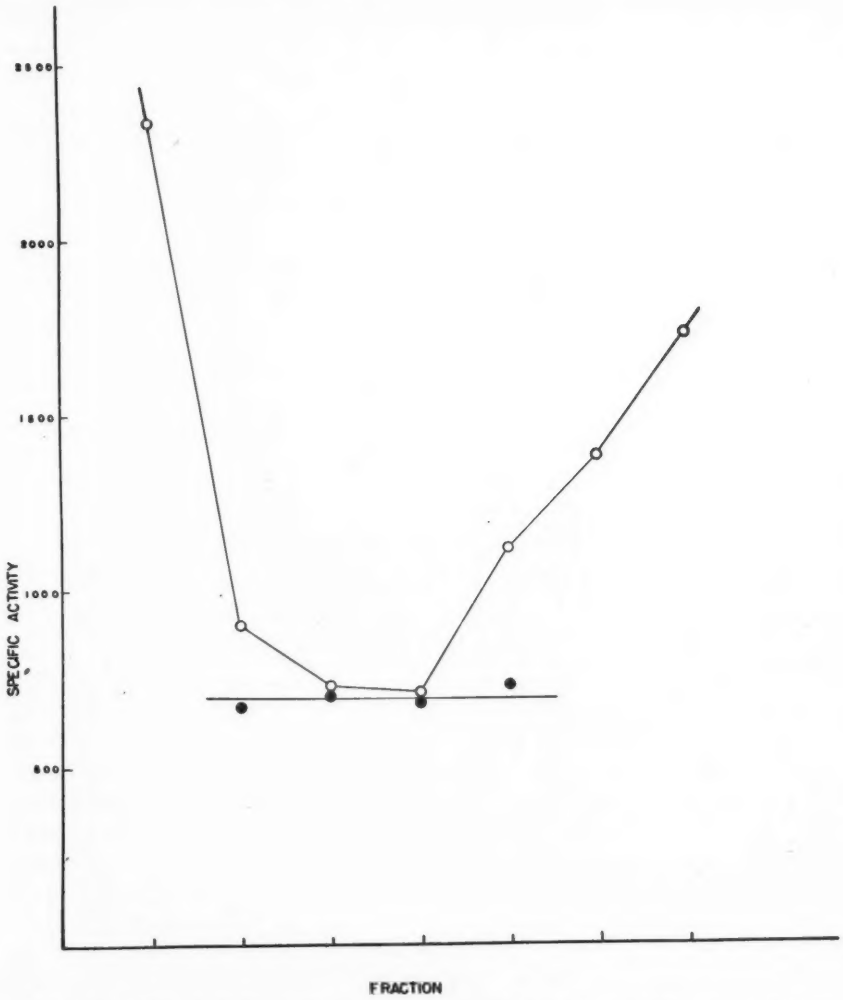

FIG. 1. Specific Activity of Twice Chromatographed Cholesterol Fractions. ○ Purified via digitonin prior to chromatography. ● Purified via the dibromide prior to chromatography.

Calculation of the per cent incorporation of labeled acetate by the perfused adrenal tumor is not meaningful in that the substrate was injected in nine portions and the circulating perfusion fluid was periodically removed after the addition of the several steroids and hormones under study.

Chromatography of the crude sterol mixture on the partition paper chromatographic system of Neher and Wettstein followed by elution and recrystallization from methyl alcohol gave a cholesterol fraction of specific activity 696 counts/min./mg.

The absence of ketosteroids in the crude sterol fraction was indicated by failure to obtain color tests with the Zimmermann test or with 2,4-dinitrophenylhydrazine on the paper chromatograms. The presence of a second sterol component on the paper chromatograms was indicated by

TABLE 2. CHROMATOGRAPHY OF PENTANE INSOLUBLE MATERIAL AND RECRYSTALLIZATION FILTRATE RESIDUE FROM DIGITONIN PRECIPITABLE FRACTION OF ADRENAL TUMOR

Elution solvent	Specific activity, counts/min./mg.
Hexane	2270
Hexane-Benzene (1:1)	906
Hexane-Benzene (1:1)	1600
Benzene	2175
Ether-Benzene (1:19)	1950
Ether-Benzene (1:3)	7700
Ether	3270
Methyl Alcohol	3060

the Liebermann-Burchard reagent. The amount of the second sterol present in the mixture is small. Characterization of the material is in process.

The presence of components of much higher specific activity than the cholesterol is evidenced in Table 2. The data are for fractions obtained from a chromatographic treatment of the digitonin precipitable, pentane insoluble material which was combined with resinous material from the recrystallization filtrates of the pentane soluble sterols. From the hexane-benzene eluted fractions an impure cholesterol fraction of specific activity 905 counts/min./mg. and melting point 141–144° was obtained. The nature of the other fractions is not known.

SUMMARY

A human adrenal tumor has been shown to utilize sodium acetate-1-C^{14} as substrate for the synthesis of cholesterol in isolated organ perfusion.

The presence of unidentified materials, coprecipitated with cholesterol by digitonin and removed from cholesterol only with difficulty, has been demonstrated in the tumor sterol fraction. Careful assay of each chromatographic fraction for radioactivity or purification via the dibromide was found to be necessary for a radiochemically pure sample.

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THE CHICK COMB RESPONSE TO ANDROGEN IN INBRED BROWN LEGHORNS

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DURING the senior author's tenure of a Fulbright Research Scholarship, Doctor Greenwood had available at the Poultry Research Centre several inbred lines of Brown Leghorns (Greenwood and Blyth, 1951; Blyth, 1952). These lines appeared desirable for detecting differences in hormone production and response as well as the relation of the endocrine system to the expression of hybrid vigor in growth rate and egg production. This report is concerned with the variability in response of the baby chick's comb to testosterone propionate.

THE ANDROGENIC COMB RESPONSE

Fussgänger (1934) emphasized the small differences in androgenic potency which could be measured by external application of the hormone to the comb of the capon. Ruzicka (1935) was probably the first to suggest that the baby chick comb response be used as an assay for androgens. Since that time there have been many research reports concerned with the possibility of the chick-comb response as a sensitive assay for androgenic potency of unknown solutions. These have been partially reviewed by Dorfman (1950).

That the sensitivity of the chick-comb response might vary according to the breed of chick used was suggested by Frank and Klempner (1937) who stated that the White Leghorn chick responds best. Dorfman (1948) demonstrated breed differences in the chick comb response. The ratio obtained by dividing the comb weight in milligrams by the body weight in grams was much greater in the White Leghorn than in Rhode Island Reds and Barred Plymouth Rocks.

Recently Campos and Shaffner (1952) demonstrated heritable differences in chick comb growth to androgen stimulation within one breed, the New Hampshire. From simultaneous injection of diethylstilbestrol and

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testosterone propionate, they estimated the heritability of chick comb size to be 0.13. These results indicated a genetic control over the chick-comb response to androgen.

There has been much controversy regarding the suitability of the baby chick's comb in androgen assay. Emmens (1939), Duff and Darby (1941), and Vallee *et al.* (1947) concluded that quantitative determination is impossible by this method. Hoskins *et al.* (1941), McCullagh and Guillet (1941) both concluded that the capon's comb was more suitable than the baby chick's comb for bioassay of androgens. A part of the variability in response from chick to chick may have been due to heritable differences in comb size of the different chicks. It was deemed of interest to determine whether there existed any difference between inbred lines and whether they were less variable than non-inbred lines.

A part of the variability in the chick response is probably due to the method of conducting and interpreting the assay. Frank *et al.* (1942) gave a detailed description of the method they finally adopted for the chick comb assay method. This and other methods have been reviewed by Dorfman (1950).

PROCEDURE OF THE TESTS

Testosterone propionate was dissolved in appropriate amounts of peanut (arachis) oil. The procedure involved the following deviations from that detailed by Frank *et al.* (1942). In exploratory tests it was observed that much of the 0.05 ml. of this oil solution invariably flooded from the comb to the less sensitive areas of the head. Rather frequently the oil ran down into the eyes eventually causing blindness in a few chicks. McCullagh and Guillet (1941) reported that no more than 0.02 ml. of oil could be applied and they preferred 0.01 ml. applied from a microsyringe. For tests comparing the response at different dosage levels 0.015 and 0.05 ml. applications were used, the former being applied by microsyringe. The down around the base of the comb was clipped to partially alleviate the problem of the oil spreading over the head. Comparisons between inbred lines were made in one test using only enough oil to cover both sides of the comb. This amounted to approximately 0.015 ml.

Treatment was begun on the second day after hatching. Daily applications were made for seven days. The chicks were killed 24 hours after the last application at 9 days of age.

The chicks were killed by cervical fracture and the comb removed with a small pair of curved scissors. By clipping the comb with the convex side of the scissors toward the head, it appeared possible to remove the comb just as precisely and with much greater rapidity than by use of the scalpel as outlined by Frank *et al.* (1942).

METHODS USED FOR COMPARISONS

It was necessary to explore methods for comparing the comb growth response to testosterone propionate application. Body size of the baby chicks from some inbred lines was significantly smaller than that of other lines. Body growth varied during the seven day treatment period. To compensate for body size at the end of the test it has been common practice (Dorfman, 1950) to express comb size as a ratio to the chick's body weight. The ratio is obtained by dividing the comb weight in milligrams by the body weight in grams. Such a procedure assumes that the comb weight bears a constant relation to body weight and that the response of the comb is dependent on the body weight of the chick.

If the hormone had been administered by subcutaneous or intramuscular injection at a site distant from the comb, it would be reasonable to assume that the amount of androgen reaching the cells of the comb might be roughly proportional to the volume of the cells in the body. However, testosterone propionate was absorbed through the surface of the comb, a localized rather than a systemic stimulation. Under these conditions the

TABLE 1. AVERAGE COMB SIZE IN NON-TREATED CHICKS OF THREE INBRED LINES AND TWO CROSSES

Matings	No. of chicks				Comb weight in mg.				Comb-body ratios*			
	2-day		9-day		2-day		9-day		2-day		9-day	
	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
B	15	13	52	61	14.6	13.0	17.2	17.5	.32	.33	.28	.28
I	18	16	56	60	8.2	7.9	9.5	9.9	.26	.26	.20	.20
I×B	15	27	9	10	10.0	8.8	10.2	13.2	.31	.32	.20	.24
B×I	28	14	10	13	11.8	10.7	13.4	12.5	.29	.31	.25	.23
W	7	9	21	35	11.0	10.0	11.3	10.7	.27	.26	.25	.24

* $\frac{\text{Comb weight in milligrams}}{\text{Body weight in grams}}$

growth response of the comb should be more directly related to comb surface during the treatment rather than to body size. Body growth might be expected to have a slight effect on comb response through a better supply of nutrients for cell growth in the larger chick. Within each inbred line, the correlation coefficient between comb and body weight of chicks is relatively low.

That initial size of the comb might be a factor affecting the variability of the comb response is suggested from the data of Dorfman (1948). Since the comb-body ratio was much larger for White Leghorns at 9 to 10 days of age, the initial comb size of this breed is probably greater than that of Rhode Island Reds or Barred Plymouth Rocks. The question arises whether the greater response of the Leghorn chick's comb may be due to a larger initial comb size rather than any greater sensitivity of their comb cells.

Data were collected for the comb weight on the second day after hatching. These are compared with the available data on comb weights at the 9 to 10 day period in Table 1. Some of the older chicks were non-treated controls from other experiments in progress during this period. Frank and Klempner (1937) demonstrated that combs of non-treated chicks were stimulated when they were intermingled with treated ones. Therefore, all control chicks were brooded in separate battery sections.

On the second day after hatching the comb weight (Table 1) was greatest for inbred line B and smallest for line I. The crosses were intermediate in comb weight, but their

comb-body ratio approached that of the larger parent (B). It will be shown later that the combs of line B grew much more rapidly under testosterone application than those of line I, and that line W was intermediate.

An analysis of variance for comb weights of B, I, I×B, and B×I at 2 days of age demonstrated that the difference between the sexes was statistically significant at the 5% level. The strain difference in comb weights was highly significant. Interaction between sex and strain was negligible and probably non-existent.

By the ninth to tenth day it will be observed (Table 1) that there were no consistent differences between the sexes for either comb weights or comb-body ratios. The differences between the comb weights of the two strains, B and I, and between either strain and the two crosses, were significant beyond the 1% level at this age as well as on the second day of age. B and I differ in actual comb weights as well as in comb-body ratios.

SOLUTION CONCENTRATION VERSUS TOTAL DOSAGE

After reviewing the literature the senior author concluded that the response appeared to be directly related to the concentration of androgen in the solution rather than to the total amount of hormone applied. The results obtained by Klempner, Hollander, and Frank (1940) would favor this conclusion. When the comb is completely covered with the oil solution at each application, it is only logical to assume that the concentration of the hormone in the solution is more important than the total amount of hormone applied.

Zondek and Sulman (1939) using 6-day old chicks, treated for 10 days, stated that a distinct difference in response was obtained between winter and summer seasons. Our tests were made during cold weather and no evidence of seasonal trends were noted.

Dorfman and Greulich (1937), and Frank, Klempner and Hollander (1938) observed that treated males had larger combs than females. McCullagh and Guillet (1941) found the comb-body ratio to average about 1.2 times greater in males than in females and used a correction factor for the sex difference. Most reports, however, indicate a similar comb or comb-body ratio for treated males and females (Dorfman, 1950). There were no statistically significant differences between the sexes at 9 to 10 days of age in our tests and, for this reason, they have been combined for this report.

A test was made by the junior author to check whether the chick comb response varies with the concentration of testosterone propionate in solution or with the total dosage applied. The average comb weights from this test are presented in Table 2. Each average is that from approximately 20 chicks, although one lot had as few as 14 chicks and another as many as 27. The 0.015 ml. daily applications were administered by means of a microsyringe.

The 0.05 ml. daily applications contained about $3\frac{1}{3}$ times as much androgen as the 0.015 ml. applications. If the comb weight response had been in proportion to the total amount (or logarithm of total amount) applied,

TABLE 2. AVERAGE COMB WEIGHTS IN RELATION TO BREEDING AND HORMONE DOSAGE

Concentration[1]	Volume[2]	Comb weight (mg.)			
		B	B×I	I×B	I
0.1	.015	40.2	30.0	21.5	20.7
	.05	36.5	22.6	20.7	17.2
0.333	.015	61.3	31.3	25.4	27.7
	.05	61.5	26.0	27.2	29.5
1.1	.015	84.6	46.0	38.1	39.0
	.05	70.0	57.2	46.4	45.1
2.0	.015		61.8		50.0
	.05		63.1		45.1

[1] Milligrams of testosterone propionate per milliliter of peanut oil.
[2] Daily application of hormone solution in milliliters.

the combs of those chicks receiving the 0.05 ml. should have been consistently larger. Considerable variability in comb weight between treatments within strains is evident from Table 2. This is due to both individual and group variability When as many as 30 to 40 individuals were used the coefficient of variation was about 35%. The number of individuals per group required to demonstrate a 20% deviation at the 1% level of significance would be 22. There was a slight but consistent depression of body weight associated with the larger quantity of oil applied. Regardless of how the comb response is expressed, it would appear the important consideration is the concentration of testosterone propionate in the solution. No increase in response is evident from increasing the quantity of solution above the 0.015 ml. daily application.

COMB RESPONSE A FUNCTION OF INITIAL COMB SIZE

Comparing the data presented in Tables 1 and 2, it is evident that the response to testosterone is in some way related to comb size of the non-treated chick and that the inbred strains differ in comb weights. Chicks of seven different inbred lines were compared using an oil solution containing 0.5 mg. of testosterone propionate per ml. of solution. The numbers of chicks involved in these comparisons are given in Table 3. The quantity of oil solution applied to each chick's comb was not measured; the procedure being to lightly cover both sides of the comb at each application. In Figure 1 there is a straight line relation between weights of treated and control combs of all lines except W. Lines with a smaller comb gave a much smaller comb growth response than those with a larger control comb weight. Even within the 7-day duration of this test, the procedure used should give a distinct advantage to the more rapidly growing comb. A regression calculated for all data except those for W demonstrates that, at

TABLE 3

Inbred line	Number of chicks		Comb-body ratios		Comb[1] ratio
	Treated	Control	Treated	Control	
R	11	15	0.53	0.23	2.4
I	41	41	0.79	0.21	3.6
S	13	14	0.80	0.24	3.5
L	16	16	0.91	0.23	4.4
W	30	30	1.33	0.24	5.8
N	32	34	1.24	0.26	5.0
B	43	45	1.69	0.31	5.8

[1] Weight of treated comb divided by weight of control comb.

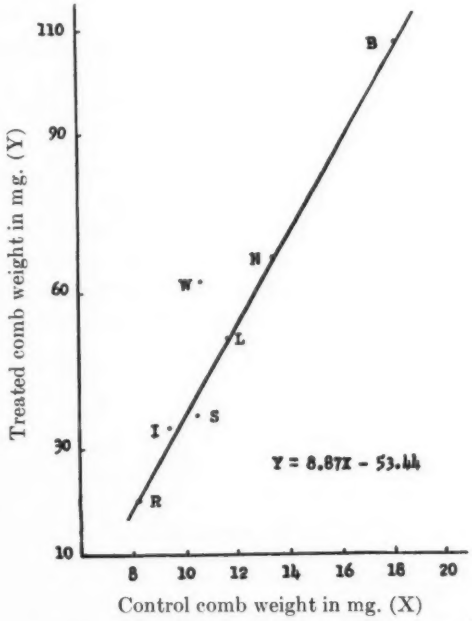

FIG. 1. Response of the combs of inbred lines to seven daily applications of a peanut oil solution containing 0.5 mg. of testosterone propionate per ml. The identity of each inbred line is indicated by a letter in close proximity to its point on the graph.

the 0.5 mg. concentration, the treated comb weight increased 8.9 mg. for each mg. increase in the comb weight of the control. We believe that inbred line W represents a strain whose comb cells are more responsive to androgen stimulation than that characterizing the other lines. The comb weight data which are expressed graphically in Figure 1 are presented as comb weight ratios and as ratios of comb weight in mg. to body weight in

grams in Table 3. The inbred lines are arranged with the smallest control comb size at the top and increasing in weight down the column. Average body weights of the groups varied from 36–37 grams for line R to 60–63 grams for line B. It will be noted that the comb-body ratios increase in magnitude in a similar manner to the actual weights as illustrated in Figure 1. When comb-body ratios of the treated and controls are compared graphically inbred lines I, L, N and B fall on a straight line. Line W is more responsive and lines S and R are respectively less responsive on a basis of the increase in comb-body ratios of the treated chicks. There was no apparent relation between comb response and amount of inbreeding of the chicks.

DISCUSSION

Since the percentage difference between treatments is greater when initial comb size is greater, it becomes important to use chicks having a relatively large initial comb size. For this reason the Leghorn breed is probably most suitable. A part of the differences in the chick comb response may be attributable to variation in initial comb size which characterizes each strain of Leghorns used by different research workers.

It is not known whether initial comb size was responsible for the heritable differences in comb response obtained by Campos and Shaffner (1952). Since the action of estrogen is antagonistic to that of androgen on the comb, their comb response data may have been confounded by the simultaneous injection of androgen and estrogen.

SUMMARY

Initial size of the chick's comb at the beginning of androgen treatment would appear to be the major factor affecting the comb response to androgen. Providing the volume of solution at each application is sufficient to cover the entire surface of the comb, comb growth varies according to the concentration of testosterone propionate in the solution and not according to the total amount applied.

Using a solution containing 0.5 mg. of testosterone propionate per ml., a straight line regression was observed between control and treated comb weights for six of seven inbred lines. At this dosage an increase of one mg. in control comb weight was associated with an increase of 8.9 mg. in treated comb weight. One inbred line appeared to deviate, giving a greater response than that expected from the performance of the other inbred lines. Most of the differences in the androgenic comb response at each dosage are due to comb size differences already present at the beginning of treatment.

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THE EFFECT OF GROWTH HORMONE ON THE NUCLEIC ACID CONTENT OF THE DEVELOPING CHICK EMBRYO[1]

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A NUMBER of investigators (Collip, 1934; Lee, 1936; Evans *et al.*, 1948; Whitney *et al.*, 1948; have observed an increase in body weight of young (sexually immature) animals receiving hypophyseal growth hormone. Similarly, growth hormone given to adult rats results in an increase in the size of all organs, but of muscles particularly (Greenbaum and Young, 1950). Recently, Hsieh *et al.* (1952), have likewise observed an increase in body weight of newly hatched chicks which had received growth hormone during embryonal development. As Russell (1953) has pointed out, it seems most likely that at least part of the action of this hormone is on some phase of the synthesis of amino acids into protein.

The investigations of Brachet (1941, 1950) and of Caspersson (1941) have shown that when there is a state of active protein synthesis, a marked accumulation of pentosenucleic acid (PNA) occurs in all cells. That growth hormone may increase nucleic acid synthesis is indicated by the investigations of DiStefano *et al.* (1952), who noted an increased concentration of nucleic acids in the liver following growth hormone administration to hypophysectomized adult rats. While in the hen's egg there is apparently little nucleic acid before development, a considerable synthesis of both desoxypentose and pentose nucleic acids occurs during embryogenesis (Plimmer and Scott, 1909; Calvery, 1928; Novikoff and Potter, 1948; Reddy, Lombardo and Cerrecedo, 1952), indicating that nucleic acid synthesis is involved in the rapid growth of the chick embryo. With the added stimulus of growth hormone and the resulting increase in body weight, it may be anticipated that alterations in nucleic acid synthesis would also occur. The present report, therefore, deals with the effect of hypophyseal growth hormone on the nucleic acid content of the chick embryo.

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EXPERIMENT

New Hampshire eggs, purchased from a local hatchery, were used throughout these experiments. Chorioallantoic injections in total dosage of 20 micrograms of growth hormone[2] were given in three equally divided doses, starting with the 13th incubation day, as reported previously (Hsieh *et al.*, 1952), and conditions of incubation were also the same as in previous experiments.

Approximately 150 eggs were used in a typical experiment; about 60 of these were used to determine nucleic acids at various stages before the 13th incubation day. The remaining eggs were divided into three approximately equal groups: two of these served as controls, one being sham operated and the second injected with saline adjusted to the same pH (8) as the hormone solution. In the early stages of embryonal development several embryos were pooled together for each determination, but from the 12th day on only one embryo was used per determination, and 3–4 such determinations were made daily for each group.

The embryos were freed of extraembryonic tissue in ice cold physiological saline and quick frozen in acetone-dry ice mixture. The frozen embryos were homogenized in ice cold 10% trichloro-acetic acid (TCA), centrifuged at 4° C., and washed with cold TCA. In embryonic stages preceding the 11th or 12th incubation day, when the embryos were still soft, homogenization was carried out in a glass homogenizer immersed in an ice-water bath; in later stages they were beaten in a previously chilled steel mill and ground in a mortar with cold 10% TCA. After removing nucleosides, nucleotides and other acid soluble compounds by cold TCA extraction, the residue was taken up in 5% TCA; the nucleic acids were extracted for 15 minutes at 90° C. (Schneider, 1945) and determined by the methods of Seibert (1940) and Mejbaum (1939), the latter as modified by Lusena (1951).

Treatment of yolk was carried out in a similar manner. The yolk was freed from the yolk sac and frozen at −20° C. A cut portion of frozen yolk was weighed on a cover slip with a precision balance and the extraction process carried out as described above.

Another group of experiments involved the nucleic acid determinations on lyophilized fat-free embryos at various incubation days. Portions of about 1.5 gm. of lyophilizes tissue were extracted with alcohol overnight and with ether for 6–8 hours in a Soxhlet apparatus. The fat-free dry tissue samples were then processed as above for determining nucleic acids.

RESULTS

Figure 1 shows that the administration of growth hormone results, by the 19th–20th incubation day, in a greater pentose nucleic acid (PNA) content per chick embryo than controls; while desoxypentosenucleic acid (DNA) shows slight, if any, increase. In Figure 2 is shown the nucleic acid concentration per unit fresh embryo weight. These results indicate that the PNA concentration reaches a maximum on the 17th incubation day in both control and experimental groups, and that the increased c ncentration resulting from growth hormone administration is apparent from the 14th day on. Alkaline saline appears also to produce a mild stimulus to PNA synthesis, but less than growth hormone.

[2] Dr. Irby Bunding of Armour & Company kindly supplied the growth hormone preparation, Lot #k-4080R.

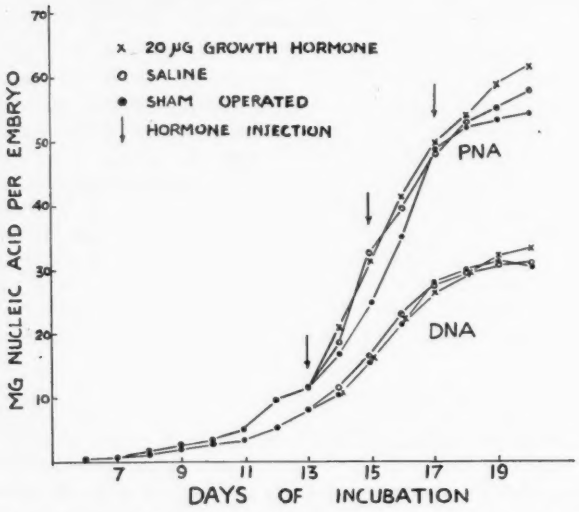

FIG. 1. Effect of growth hormone on the nucleic acid content of chick embryo

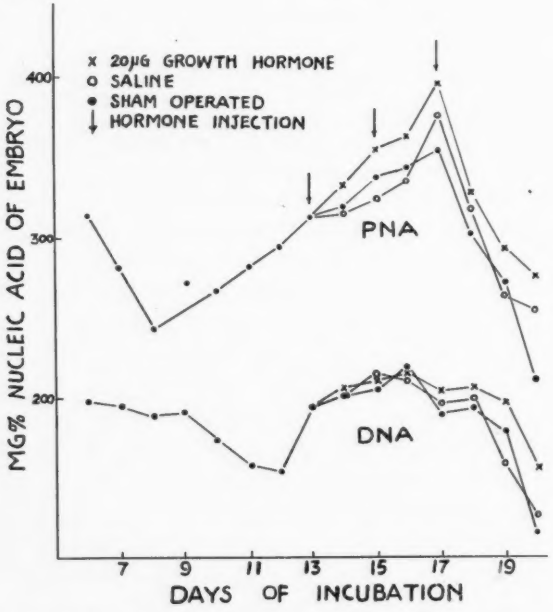

FIG. 2. Effect of growth hormone on the concentration of nucleic acids of chick embryo (fresh tissue).

The curves showing DNA concentrations are somewhat different. While it also increases in concentration for some time during the latter part of embryogenesis, it does not reach a distinct peak, but holds an approximate maximum between the 14th and 18th incubation days. However, as in the case of PNA, the DNA concentration falls during the last few days of the embryogenic period. The concentration of DNA is about the same in the two control groups during the last few incubation days, and is higher in the experimental group.

Figure 3 shows the changes in nucleic acid concentrations per unit fat-free dry embryo tissues between the 14th and 20th incubation days. Contrary to the wet weight results, there is essentially a continuous decline in the concentrations of both nucleic acids, except for a moderate rise in PNA in both groups at about the 18th incubation day; this rise is more marked in experimental embryos. Growth hormone-injected embryos exhibit no difference in DNA concentration from saline controls, but show consistently higher PNA levels after the 17th day of incubation. Before this time, however, the relationship is reversed and saline controls show slightly higher PNA concentrations than experimental animals.

In order to eliminate the possible influence of faulty selection of eggs an

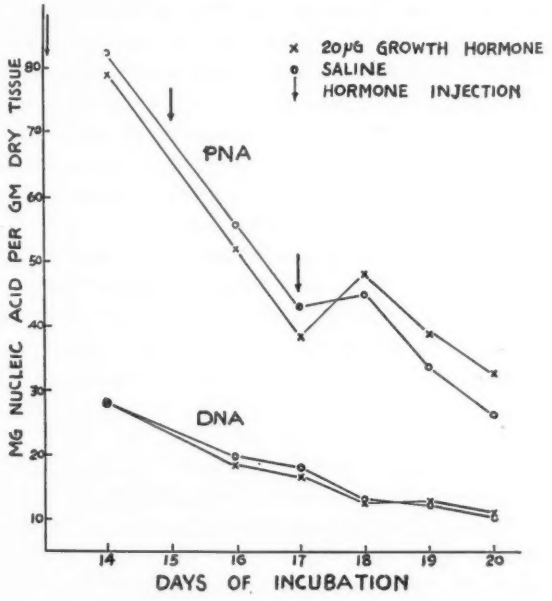

FIG. 3. Effect of growth hormone on the nucleic acid concentration of fat-free dry chick embryo tissue.

attempt was made to compare egg weight with the weight of the embryo in a series comprising a large number of eggs (122 control, 96 experimental). It was thought that the selection of embryos of similar weight in the three groups at comparable stages in the experiment might clarify the validity of the observed differences in nucleic acid concentrations. However, it was not possible to accomplish this by selecting eggs on a weight basis, since the latter does not change significantly as the weight of the embryo increases. While there is a general tendency for hormone treated embryos to weigh more than saline controls, this difference is of doubtful significance. The weight differences observed previously in newly hatched chicks (Hsieh *et al.*, 1952) must therefore occur during the last one or two days of incubation.

We have been unable to detect either PNA or DNA in the yolk by the methods used here. Yolk homogenate has failed to show any characteristic absorption at 260 mu, nor the typical colors by the diphenylamine and orcinol reactions.

DISCUSSION

These results indicate a greater total quantity of PNA per growth hormone treated embryo during the last 3–4 incubation days; however, there is no difference in the total quantity of DNA between control and experimental embryos. On the other hand, when the data are calculated as concentration per unit fresh tissue weight, there appears to be an increase in both nucleic acids in growth hormone injected eggs. The effect of growth hormone in increasing PNA concentration during the latter part of the embryogenic period is further substantiated by the determinations of nucleic acid concentrations of fat-free dry tissue, but such an analysis fails to reveal any difference in DNA concentration.

The difference in PNA concentration between growth hormone injected and control embryos on the 17th incubation day when there is no difference in total quantity per embryo might perhaps be accounted for on the basis of changes in the content of fat, water, or both. Furthermore, fluctuations in the concentrations of both nucleic acids between the 7th and 20th incubation days, when there is a steady rise in total quantity per embryo, may also be due to changes in fat and/or water content. Such a possibility is further indicated by the observation that there is a steady fall in the concentrations of both nucleic acids when determined on fat-free dry tissue, while there is an apparent increase in the concentrations of these substances on a wet weight basis.

Such fluctuations in water and fat content of the chick embryo may also explain differences in nucleic acid concentrations as observed by Novikoff and Potter (1948), as well as Cavallero *et al.* (1952) on a wet weight basis,

and by Reddy *et al.* (1952) whose analyses were on dried tissues. In general, our wet weight determinations agree with the former and the fat-free dry weight determinations approximately with the latter. The only difference from the observations of Reddy *et al.* (1952) deserving comment is the slight rise in PNA concentrations in controls and the larger increase in hormone injected embryos between the 17th and 18th incubation days, as shown in Figure 3. Repeated analyses have substantiated this difference for which we have as yet no explanation. It is pertinent, however, that it occurs at a time when a sharp drop in mitotic rate is initiated (Blumenthal *et al.*, 1953).

It appears therefore that while growth hormone administration to the chick egg results in a greater PNA content and concentration than in controls, certain apparent discrepancies between the curves showing concentrations, as well as certain fluctuations in the quantity of nucleic acid per unit wet tissue weight in normal animals may be due, in large part, to shifts in water and/or fat content.

Of pertinent interest in avian embryogenesis is the present observation that nucleic acids apparently are not synthesized in the yolk, but the latter may furnish substances for synthesis of DNA and PNA elsewhere in the embryo.

SUMMARY

The total content and concentrations of DNA and PNA of the chick embryo and yolk in normal and growth hormone injected hen's eggs have been determined. The data indicate that hormone administration results in an increase in total PNA during the late stages of embryogenesis. When the data are calculated on the basis of concentration per embryo on a wet tissue basis and of quantity per unit fat-free dry embryo tissue, the hormone treated animals show greater concentrations of PNA than controls. The concentration of DNA does not appear to be appreciably influenced by growth hormone. The possible role of water and fat content in evaluating the significance of these and similar data of other investigators is discussed.

There is no detectable nucleic acid in the yolk during embryonal development in either control or experimental groups.

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THE MEASUREMENT OF ESTROGEN MIXTURES BY DIFFERENTIAL FLUOROMETRY[1,2]

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THE fluorometric measurement of estrogens has become a widely used procedure since the publications of Jailer (1948), Bates and Cohen (1950), and others. More recently, the nature of the reaction of strong acids with natural estrogens for the production of fluorescence has been investigated systematically (Goldzieher, Bodenchuk and Nolan, 1952); it was verified that sulfuric acid under the conditions advocated by Bates and Cohen and with excitation by the 436 mμ line of the mercury spectrum produced maximum fluorescence. However, the method still presents a number of major technical obstacles.

The preparation of reagents sufficiently pure for fluorometric work has received scant attention in the literature, yet with the increased sensitivity of modern photomultiplier fluorometers and with improved methods for the development of fluorescence, reagent contamination (causing either non-specific fluorescence or quenching of fluorescence) has often become the limiting factor in the sensitivity of the method.

The necessity for removing contaminants from the biological extract as well has become amply clear. In addition to the substances which interfere with the production of fluorescence, one must also eliminate those which produce discoloration of the solution and thus cause self-absorption of the fluorescent radiation. There is good evidence that countercurrent distribution (Slanuwhite *et al.*, 1951) and chromatographic procedures (Swyer and Braunsberg, 1951) can accomplish this, and the problem is being studied intensively at a number of laboratories. Consequently the present study does not encompass this aspect of the problem.

Furthermore, it is evident that measurement of fluorescence must in some way be related to the total as well as the respective amounts of estrogens in a biological mixture. As estrone, estradiol-17β and estriol have significantly different fluorescence energies and spectra (Goldzieher *et al.*,

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[2] Read by title at the Annual Meeting of the Endocrine Society, June 1953.

[3] Present address: Southwest Foundation for Research and Education, San Antonio, Texas.

1952), a single broad-band determination of fluorescence energy does not yield much specific information as to the nature and amounts of estrogens present. Separation of the estrogens for individual measurement can be accomplished by countercurrent distribution, but this procedure is not routinely available, at least at present. Recent work (Bitman and Sykes, 1953) indicates that successful chromatographic methods may be in the offing. A third possible approach was indicated in our previous studies, which revealed that it might be possible to utilize the differences in the fluorescence spectra of the estrogens to effect an optical differentiation of the components of such a mixture; this, together with certain important difficulties related to the reproducibility of the fluorescence reaction is the subject of the current investigation.

MATERIALS AND METHODS

Apparatus.—As fluorescence is particularly sensitive to contaminants, great care must be observed in the preparation of glassware as well as in the selection of reagents. After routine cleaning, it is advisable to immerse all glassware in hot 50% nitric acid for at least 30 minutes, followed by thorough washing with distilled water and drying in an incubator or other space from which room dust can be excluded.

Reagents.—Our studies have confirmed the observations of Engel *et al.* (1950) with regard to the influence of solvents. Failure to use a solvent for the estrogen prior to the addition of acid results in a loss of reproducibility. The 1:20 alcohol-toluene mixture of Engel gave the best results, while alcohol alone yielded consistently less fluorescence. Reagent grade absolute alcohol was allowed to stand for a week with m-phenylenediamine and was then distilled in an all glass system. Toluene also has been found to vary in quality. One lot (Baker reagent grade) was simply redistilled; less satisfactory samples may have to be washed with 65% sulfuric acid, 50% nitric acid, and then numerous times with distilled water before drying and redistilling. However, the blank fluorescence of our best lot of toluene was not decreased further by such treatment.

It has become increasingly evident that the quality of sulfuric acid used is of paramount importance. Both Bates and Cohen and Engel *et al.* have found that linearity of calibration depends upon the commercial source of the acid. Furthermore, the sulfuric acid itself accounts for most of the blank fluorescence and is therefore the limiting factor in the sensitivity of the method. After experiencing considerable difficulty with various lots of acid, the necessity of redistillation became clear. Widespread opinion to the contrary, this has proved to be an extremely simple procedure if concentrated acid is vacuum distilled in the apparatus shown in Figure 1. Dilute acid (sp. g. 1.55) will bump severely, but no difficulty has been experienced with duPont acid of specific gravity 1.884.

The distilling vessel is an ordinary 1-liter round bottom Pyrex flask heated by a hemispherical electric mantle. It may be charged with 500 to 700 ml. acid at one time. The distilling head and receiver must be annealed with care. The receiving flasks are 250 or 500 ml. round bottom Pyrex flasks. All joints are sealed with Dow-Corning high vacuum silicone grease. The trap is immersed in a dry ice-alcohol mixture and a Welch or similar high vacuum mechanical pump is used to exhaust the system. After evacuation the current to the heating mantle is increased until the sharp vertical edge of the condensing column of acid is in the midportion of the up-angled side-arm of the distilling head. Distillation will proceed rapidly without any visible disturbance of the surface of

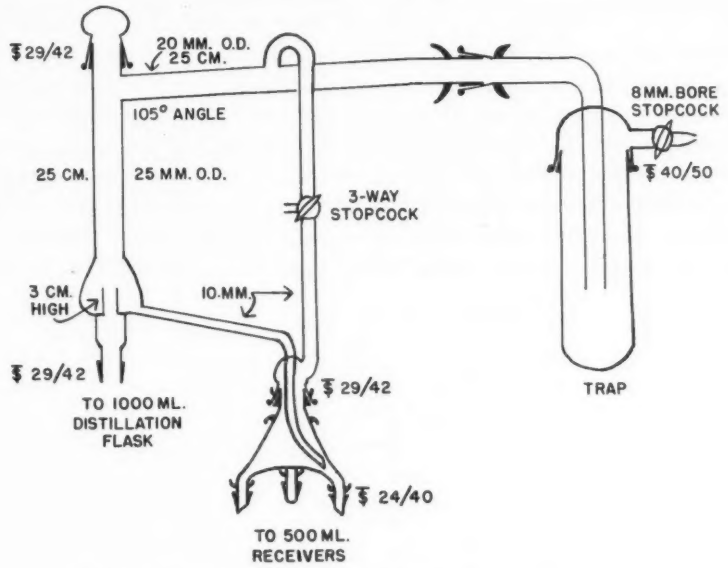

FIG. 1. Pyrex distillation head and receiver used in the vacuum distillation of sulfuric acid.

the heated acid. The first cut of 150 ml. or so contains nearly all the extraneous fluorescent material. A center cut of about 400 ml. yields acid of excellent characteristics. The distillation is so smooth that we have dispensed with temperature or pressure measuring devices and simply distil at maximum vacuum at a predetermined setting of the voltage regulator of the heating unit. Bumping will occur only if the vacuum is disturbed by plugging of the trap, etc. The blank fluorescence of the resultant acid is, at most, only half that of the best commercial product we have been able to obtain. The distilled acid is then made up to 90 and 65% concentrations according to the technique of Bates and Cohen.

Method.—Appropriate amounts of pure estrone, estradiol-17β and estriol, dissolved in absolute alcohol, were pipetted into carefully cleaned 1 × 9 inch test tubes. The volume of alcohol was made equal in all tubes and the alcohol was then evaporated in vacuo at room temperature. Duplicate tubes were prepared in all cases.

The estrogen was then redissolved in 0.2 ml. of 1:20 alcohol-toluene with slight warming. 1.0 ml. of 90% sulfuric acid was added, the tubes were immersed in a water bath at 80°C. for 10 minutes, and final dilution was carried out with 5.0 ml. of 65% acid. The fluorescence of the product was stable for at least 24 hours.

Measurements were carried out in a Farrand fluorometer. This instrument uses a mercury vapor light source; the 436 mμ line was isolated by means of a Corning 5113 and 3389 filter combination, while the 405 mμ line was obtained with a Corning 3060-5870-4308 or a 3060-9863 combination. The resultant fluorescence is collimated in this particular instrument, thus permitting the use of interference-type secondary filters. The exact specifications of these secondary filters are discussed below.

RESULTS

Figures 2A and 2B illustrate portions of the spectral fluorescence curves of 5 γ/ml. of estrone, estradiol-17β and estriol obtained upon development with sulfuric acid and excitation with the 436 and 405 mμ mercury lines (3). It is evident that the 436 mμ line (being closest to the region of maximum absorbance at 450–490 mμ) excites maximum fluorescence. Furthermore, the fluorescence intensities of estrone and estradiol-17β are equal at 470 mμ. A narrow-band filter at this wavelength should therefore give equal values (on a weight basis) for estrone and estradiol-17β. In actual

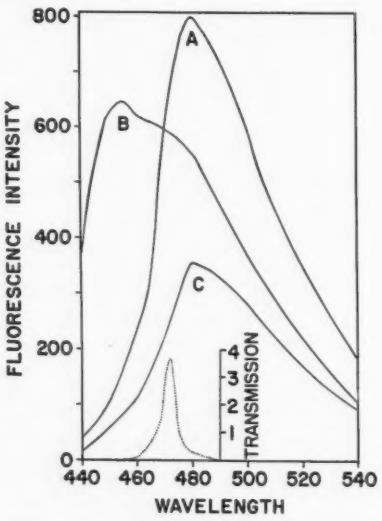

FIG. 2A. Fluorescence spectra of estrone (A), estradiol-17β (B) and estriol (C) on excitation with the 436 mμ mercury line. Dotted curve (ordinate insert is per cent transmission) shows transmission characteristics of 470 mμ filter described in text.

practice this will be complicated by the fact that the spectra are asymmetrical on either side of 470 mμ, particularly at the shorter wavelengths. The shape of the filter transmission curve, especially in the 455–465 mμ region, will therefore largely determine how closely the fluorescence output of these two estrogens can be equalized. Some difficulty was encountered in obtaining a filter to meet these exacting requirements; the best interference filter to date has a peak transmission of 3.8 per cent at 472 mμ and 0.0 transmission at 458 and 488 mμ, as shown in Figure 3. It may be seen that the fluorescence intensities of estrone and estradiol-17β differ by only 10 per cent. The value for estriol, as may be anticipated from the spectral curve, is considerably lower. This filter system, therefore, will

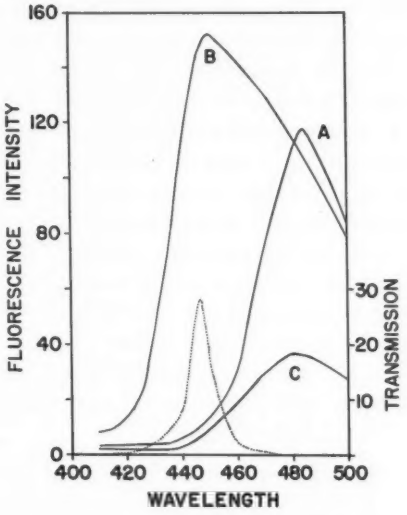

FIG. 2B. Fluorescence spectra as above, with excitation by the 405 mμ mercury line. Dotted curve shows transmission characteristics of Corning 3389 plus 446 mμ interference filter. Right-hand ordinate in per cent transmission.

give a reasonably accurate estimate of the total amount of estrogen (on a weight basis) in an estrone-estradiol-17β mixture. It does not appear from the three spectra that a filter system can be devised, at least in the region studied, which will select equivalent fluorescence energies for all three estrogens. As a consequence, one must consider inclusion of a step in the isolation procedure which will separate estriol from the other two estrogens. Friedgood *et al.* (1948) have proposed one such method, and

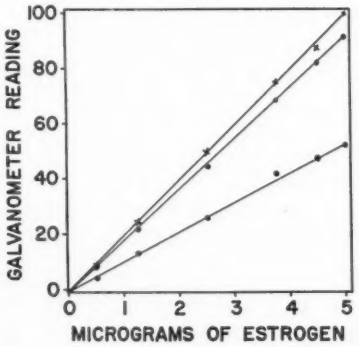

FIG. 3. Calibration curves of pure estrogens measured at 470 mμ and 436 mμ excitation. Estrone is indicated by X, estradiol-17β by ○, estriol by ●.

Engel has calculated that the original Mather procedure (two partitions between benzene and 0.3 M sodium carbonate, with backwashing) will accomplish a quantitative separation.

The spectral curves also show that the fluorescence intensity of estradiol-17β remains high at the lower end of the spectrum, while those of estrone and estriol drop off rapidly. It may be anticipated that at these shorter wavelengths, in the region of 440 mμ, an adequate optical separation can be achieved. However, accurate measurements in this region are complicated by the fact that scattering and surface reflections may deflect substantial amounts of the 436 mμ exciting radiation toward the secondary filter. As no currently available filters will exclude completely the 436 mμ line while transmitting an adequate fraction of the 440 mμ fluorescence, false high values or high blank readings will result. However, use of the 405 mμ mercury line gives an adequate separation of the exciting and secondary radiation and does not alter desirable differences in the fluorescence spectra (Figure 2B). Figure 2B shows the transmission curve of a suitable interference filter with the peak at 446 mμ preceded by a Corning 3389 glass, as well as the result obtained with this system. Estrone and estriol are now only one tenth as fluorogenic, weight for weight, as estradiol-17β. This arrangement, therefore, permits the selective measurement of estradiol-17β in a binary or ternary mixture with reasonable specificity. By selecting a filter of intermediate characteristics, transmitting between 450 and 460 mμ, it is theoretically possible to measure the fluorescence energies of estradiol and estrone in the same ratio as their biological activities.

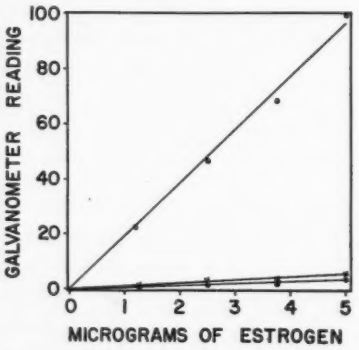

FIG. 4. Calibration curves of pure estrogens measured at 446 mμ with 405 mμ excitation. Estrone is indicated by X, estradiol-17β by ○, estriol by ●.

The additivity of fluorescence.—In consequence of these findings it becomes important to know whether different estrogens heated simultaneously with sulfuric acid react in an independent, quantitative manner or whether interaction or quenching occurs. To study this question, three

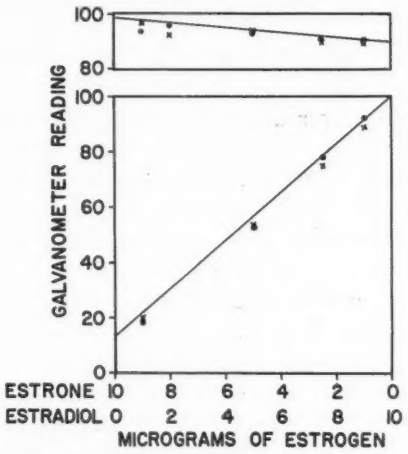

FIG. 5. The additivity of estrogen mixtures measured with 436–470 mμ system (*above*) and 405–446 mμ system (*below*). Solid line indicates theoretical values derived from calibration curves. ● indicates measured values of mixtures of estrone and estradiol-17β combined and developed simultaneously. X indicates measured values of estrogens developed separately and combined after development.

types of samples were prepared. The first group comprised standard solutions of single estrogens from which calibration curves were constructed and from these curves the theoretical total fluorescence of a given mixture could be calculated. In the second group, combinations of 2 or 3 estrogens over a wide range of proportions (from 1:10 to 10:1) were prepared and developed in the usual manner. In the third group, solutions of the individual estrogens were combined *after development* so that the final concentrations in these mixtures were identical with those of group 2. A typical result (Fig. 5) shows that the fluorescence of groups 2 and 3 agrees very closely with the theoretical calculations, indicating that no significant interaction or quenching occurs during or after the sulfuric acid development of a mixture of pure estrogens. Table 1 shows a similar experiment carried out with a ternary mixture, and again it is found that measurements agree well with theoretical expectations.

TABLE 1. THE ADDITIVITY OF TERNARY ESTROGEN MIXTURES. COMPARISON OF ACTUAL WITH COMPUTED FLUORESCENCE INTENSITY

Estrogen			Fluorescence of mixture (436–470 mμ system)	
Estradiol in μg.	Estrone in μg.	Estriol in μg.	Calculated	Measured
3.0	1.0	1.0	77	76
1.0	3.0	1.0	86	81
1:0	1.0	3.0	64	61

DISCUSSION

The evidence just presented indicates that selection of appropriate secondary filters can yield valuable information in the measurement of estrogen fluorescence in mixtures. The choice of a suitable filter involves several considerations. Above all, the secondary filter must be *completely* opaque to the primary radiation. This is of paramount importance when the fluorescence energy is low, and requires exact knowledge of the transmission spectrum of the given filter, with particular attention to the "toe" of the curve. A fraction of a per cent transmission of the exciting radiation may be sufficient to vitiate measurements with solutions of very low fluorescence. The choice of a wide or narrow-band (interference) filter depends largely upon the specific circumstances. In a situation involving the fluorescence of a single compound with a very low blank or nonspecific fluorescence, the wide-band filter has the advantage of transmitting a greater total energy and consequently permitting measurements at much greater dilution. Narrow band filters transmit much less total energy and may require a photomultiplier type of instrument. However, in some instances linearity can be achieved with narrow band filters where other types fail. Furthermore, a troublesome nonspecific fluorescence may be largely eliminated if its spectrum differs from that of the test substance. This is specifically true in the determination of estrogens from extracts of human urine. Spectral fluorescence curves of the residue obtained by extracting urines from children under 4 years of age by the method of Engel *et al.* (1950) show that the fluorescence energy is evenly distributed, at a very low level, over the entire measured spectrum. In older individuals, however, an appreciable nonspecific fluorescence begins to appear with a maximum in the 510–525 mμ region. An interference filter which will cut off in this region is clearly useful in decreasing the specific-to-nonspecific fluorescence ratio.

Lastly, the nature of the fluorescence spectrum of the substance or substances under study dictates the exact requirements for the band width and transmission peak of the filter, as shown by our data. It may be pertinent to mention that commercially available interference filters show great variation in quality. Several failures with the fluorescence technique, where the only fault was an interference filter of unsuitable characteristics, have come to our attention.

It may also be pointed out that a discontinuous source of energy, such as mercury vapor, greatly simplifies the choice of filters and the problem of excluding scattered incident radiation.

Biological applications.—There is ample evidence that a urinary estrogen extract obtained, for example, by the extraction procedure of Engel *et al.* cannot be used for fluorometric measurements without further purification

except at extremely high estrogen levels where blank fluorescence, self-absorption or quenching can be eliminated by sufficient dilution. A chromatographic purification which separates the estrogens from chromogens and other phenols should be adequate for this purpose.

Even if chromatography does not perform a quantitative separation of the estrogens from each other, an index of the degree of contamination of one estrogen with another can be obtained by the type of differential fluorometry just described. An unknown preparation, if pure, will maintain a constant ratio of fluorescence intensity to that of a known pure standard preparation despite changes of the secondary filter (for example from 470 to 440 mμ). The sensitivity of this technique, for which only minute amounts are required, can be determined in any given situation by appropriate calibration curves of the substances involved or by the preparation of model mixtures.

If heating with sulfuric acid produces nonspecific chromogens, a further complication is introduced into the measurement of fluorescence. The optical geometry of most fluorometers is such that a correction factor based on the absorbance of the solution, measured at any given wavelength or even through the secondary fluorescence filter itself, cannot readily be computed. Increased dilution (where the concentration of the test substance permits) or further purification appear to be the alternatives.

SUMMARY

1. Purification of solvents and sulfuric acid used in the quantitative determination of estrogen fluorescence is described.

2. Based on the fluorescence spectra of crystalline estrone, estradiol-17β and estriol, conditions have been defined for the total and differential measurement of estrogen mixtures. At 470 mμ estrone and estradiol-17β have almost equal fluorescence, while at 440 mμ estradiol-17β has ten times the fluorescence of estrone or estriol.

3. Criteria for the selection of satisfactory filter systems are outlined.

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SELECTIVE DEPLETION OF THE ADRENALINE CONTENT OF THE SUPRARENAL GLAND BY THE INJECTION OF INSULIN IN THE CAT[1]

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IT HAS recently been shown that the suprarenal medulla contains not only adrenaline but also noradrenaline. The quantity of the latter substance varies considerably from one species to another, while the variation in content from one animal to the other within the same species is very small. A fundamental question, is whether adrenaline and noradrenaline are two independent hormones, which are selectively secreted from the suprarenal medulla under different conditions. In attempts to answer this question the effect of insulin induced hypoglycemia on the suprarenal content of catechols (adrenaline plus noradrenaline) has been previously studied. For these experiments, rats (Burn, Hutcheon and Parker, 1950; Outschoorn, 1951, 1952, Hökfelt, 1951) and rabbits (West, 1951; Hökfelt, 1951) were used. A decrease in the adrenaline content was invariably observed following the injection of insulin; a complete depletion of adrenaline was noted by Hökfelt. The small suprarenal content of noradrenaline in the animals investigated before has, however, caused difficulty in judging the effect of hypoglycemia. For this reason similar experiments have now been performed in the cat, whose suprarenals contain relatively large quantities of both noradrenaline and adrenaline.

METHODS

Cats of both sexes were used; the weight varied between 2.5–4.0 kg. On the day preceding the experiment the animals received food (mainly fish and milk) in ordinary quantities; during the course of the experiment neither food nor drink were permitted. Regular insulin[2] (40 I.U./ml.) was given subcutaneously as single injection in a dose amounting to 10 I.U./kg. body weight (b.w.) during December and January; during February–April the dose was gradually decreased to 2 I.U./kg. b.w. to avoid insulin shock.

At various times after the administration of insulin the adrenals were extirpated under intraperitoneal anaesthesia (*Citodon-Na*,[3] 50 mg./kg. b.w.). After weighing, the adrenals

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[2] *Insulin-Vitrum*, kindly supplied by professor E. Jorpes, M.D., Vitrum Company, Stockholm, Sweden.

[3] *Citodon-Na* (=N-methylcyclohexenylmethylbarbiturate) was kindly supplied by Leo Company, Hälsingborg, Sweden.

were ground in a mortar containing sand and 10 ml. acid alcohol (1.25 ml. 1-N H_2SO_4 /100 ml. 95% ethyl alcohol). Extracts were then refrigerated (+4° C.) for 1 hour. Following filtration of the extracts, and reextraction of the tissues with another 10 ml. acid alcohol, the alcohol extracts were evaporated under nitrogen, over a boiling water bath. When the alcohol extract had been concentrated to about $\frac{1}{3}$ of its original volume, 2 ml. distilled water containing 1.25 ml. 1-N H_2SO_4/100 ml. was added and evaporation continued to a volume of about 1 ml. This highly concentrated extract was then made up to a final volume corresponding to 40–50 mg. tissue per ml. and the pH corrected to about 3.0 by the addition of 0.25-N NaOH. The extracts were shaken with 1 volume of ether *pro analysi* to remove the lipids and left in a separation funnel for 8 hours. The amounts of catechols thus obtained in the water phase were then determined both colorimetrically (Euler and Hamberg, 1949) and biologically according to Euler (for details: see Hökfelt, 1951).

RESULTS

In the control cats (Table 1) the suprarenal content of both catechols in absolute figures was significantly different depending on the sex (male 1.14 μg. ± 0.139 adrenaline and 0.96 μg. ± 0.119 noradrenaline; female 0.54 μg. ± 0.028 adrenaline and 0.55 μg. ± 0.071 noradrenaline). The percentage of adrenaline of total catechols, however, was approximately the same in both sexes (male 54.4% ± 2.21, female 50.4% ± 2.48).

TABLE 1. ADRENALINE AND NONADRENALINE CONTENT (μG./MG. TISSUE) OF THE SUPRARENAL GLAND IN THE CAT AT DIFFERENT INTERVALS FOLLOWING THE INJECTION OF INSULIN (2–10 I.U. PER KG BODY WEIGHT GIVEN SUBCUTANEOUSLY.) THE FIGURES ARE DIVIDED INTO TWO GROUPS ACCORDING TO SEX

Hours after injection of insulin	Sex	Adrenaline	Noradrenaline	Adrenaline in per cent of both catechols
7 controls	male	1.14 ± 0.139	0.96 ± 0.119	54.4 ± 2.21
1.5	male	0.73	0.88	45.3
3.0	male	0.61	1.06	36.5
6.0	male	0.35	1.09	24.3
8.0	male	0.33	0.98	25.2
8.0	male	0.27	1.00	21.2
0.9	male	0.11	1.02	9.7
5 controls	female	0.54 ± 0.028	0.55 ± 0.071	50.4 ± 2.48
3.0	female	0.42	0.49	46.1
3.0	female	0.25	0.69	26.6
6.0	female	0.27	0.51	34.6
6.0	female	0.23	0.50	31.5
6.0	female	0.17	0.67	20.2
8.0	female	0.10	0.51	16.4
8.0	female	0	0.52	0
8.0	female	0.13	0.53	19.7
8.0	female	0.19	0.71	21.1
9.0	female	0.08	0.62	11.5
9.0	female	0.10	0.59	14.5
9.0	female	0.03	0.60	4.8

The difference in catechol content between the right and the left suprarenal was also studied in 5 normal cats (Table 2). In no case did the difference of catechols in absolute figures vary more than 10%; the adrenaline

content expressed in per cent of both catechols differed less than 5%.

The injection of insulin was followed by a continuous decrease of the adrenaline content of the suprarenal and after nine hours only 5–19% of the normal amount was found (Table 1 and Fig. 1). In all cases the absolute noradrenaline values remained within normal limits.

TABLE 2. COMPARISON BETWEEN THE CATECHOL CONTENT OF THE RIGHT AND THE LEFT SUPRARENAL IN THE SAME ANIMAL

Sex	Adrenal weight in mg.		Adrenaline μg./mg. tissue		Noradrenaline μg./mg. tissue		Adrenaline in per cent of both catechols	
	Right	Left	Right	Left	Right	Left	Right	Left
male	132	133	1.33	1.31	1.31	1.36	50	49
male	132	139	1.69	1.69	1.29	1.19	57	59
female	192	192	0.65	0.68	0.65	0.72	50	49
female	173	179	0.55	0.55	0.54	0.52	51	52
female	197	201	0.57	0.57	0.79	0.73	42	44

DISCUSSION

Burn *et al.* (1950) injected 0.2 I.U. insulin per 100 gm. body weight in the rat and observed a decrease of the adrenaline content of approximately 50%. Hökfelt (1951) found a total depletion of the adrenaline content in the rat adrenal nine hours after the administration of 10 I.U. insulin per 100 gm. body weight and Outschoorn (1951, 1952) using 1 I.U. insulin per 100 gm. body weight observed a decrease of about 85% for the adrenaline in the suprarenal of the same animal, 16 hours after the injection. None of these authors reported any significant change in the suprarenal noradrenaline content at the time intervals mentioned, although Outschoorn noted a tendency to a decrease.

The figures presented in this work clearly show that the injection of insulin in the cat is followed by a depletion of the adrenaline content of the suprarenal without a concomitant fall in the amount of noradrenaline (Fig. 1). To obtain this effect it appears, however, necessary to avoid profound insulin shock.

If increasing doses of insulin (10–20 I.U. per kg. b.w.) are injected in the cat severe persistent insulin shock is produced. This can be temporarily overcome by the subcutaneous administration of 5 ml. 2% glucose solution. In this way a cat can be submitted to repeated episodes of deep insulin shock for 8–20 hours which is followed by a depletion of both the adrenaline and the noradrenaline content of the adrenal gland (Hökfelt, unpublished). It is of interest that 24–32 hours following the injection of insulin in the rat a significant decrease not only of the adrenaline but also of the nor-

adrenaline content of the suprarenal was observed by Hökfelt (1951), and that it was necessary also in these experiments to give glucose to counteract severe insulin shock.

The selective depletion (and secretion) of adrenaline from the adrenals following the administration of insulin as described in this paper probably indicates that adrenaline plays a role as a specific *metabolic* hormone. As

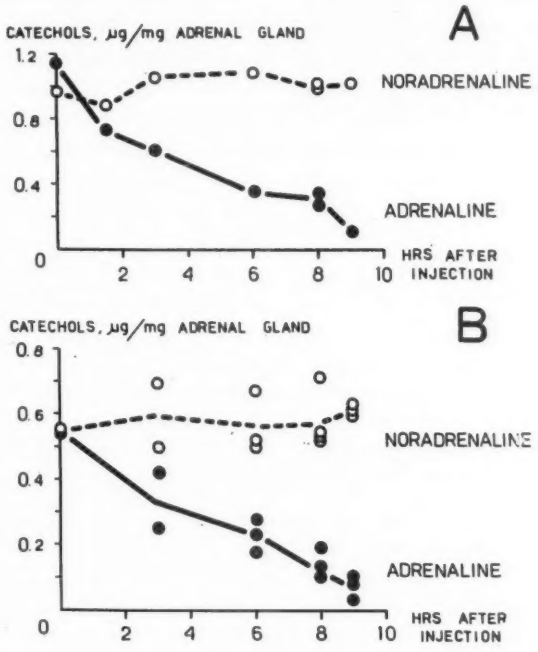

FIG. 1. Graphic representation of the adrenaline and noradrenaline values found in the suprarenal gland of the cat at different periods of time following the injection of insulin. A: male cat; B: female cat.

to the noradrenaline a number of investigators have shown that this substance under various experimental conditions is present in the suprarenal vein in quantities often exceeding those of adrenaline (Bülbring and Burn, 1949 a; Brauner, Brücke and Kaindl, 1949; Brauner, Brücke, Kaindl and Neumayer, 1951; Kaindl and Euler, 1951; Dunér, 1953). This might support the assumption that noradrenaline plays a role as a hormone *per se*, which, of course, does not exclude the possibility that noradrenaline also acts as a precursor to adrenaline as first shown by Bülbring (1949) and Bülbring and Burn (1949 b).

SUMMARY

1. The catechol content of the suprarenal gland in the normal cat has been found to be the following, expressed in μg./mg. tissue: *male* 1.14 μg. ± 0.139 adrenaline and 0.96 μg. ± 0.119 noradrenaline, adrenaline in per cent of adrenaline plus noradrenaline 54.4% ± 2.21; *female* 0.54 μg. ± 0.028 adrenaline, 0.55 μg. ± 0.071 noradrenaline, adrenaline in per cent of both catechols 50.4% ± 2.48.

2. Nine hours after the injection of insulin (2–10 I.U. per kg. body weight) in the cat, practically all the adrenaline of the suprarenal had been depleted, while there was no change in the noradrenaline content.

3. The results of this study favour the assumption that adrenaline and noradrenaline are two independent adrenal hormones which are selectively made use of under different conditions.

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EFFECT OF HYPOPHYSECTOMY, DDD TREATMENT, AND SURGICAL TRAUMA ON THE OXYGEN CONSUMPTION OF THE VARIOUS ZONES OF THE ADRENAL CORTEX[1]

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PREVIOUS reports (Carpenter *et al.*, 1946; Tepperman, 1950; Nichols and Little, 1951; Saffran *et al.*, 1951; Ferstl *et. al.*, 1951; Overbeek and Van der Vies, 1952) have indicated that administration of ACTH to adrenal tissue *in vitro* causes an increase in oxygen consumption and a depletion of ascorbic acid. It has also been reported (Nichols and Little, 1951) that tangential slices consisting predominantly of zona glomerulosa and of zona fasciculata have different rates of oxygen consumption and react differently to ACTH administered *in vitro*. Some writers hold the view that the inner zones of the adrenal cortex are under influence of the pituitary (ACTH) while the outer zones are not. This view is supported by the observations that the inner zones undergo a more profound atrophy after hypophysectomy. In view of the foregoing one might suspect that the various zones of the cortex would become resistant or sensitized to ACTH *in vitro* when taken from an animal a few days after hypophysectomy (hypostimulation) or after stress (surgical trauma—endogenous stimulation by its own ACTH). This paper is a report of such study and also of the response of glands taken from an animal whose cortex is undergoing atrophy from DDD. This latter substance (2,2-bis(parachlorophenyl)-1,1-dichloroethane) brings about an atrophy similar to that of hypophysectomy. The results are compared with those of glands from normal animals reported previously (Nichols and Little, 1951).

MATERIALS AND METHODS

The source of the adrenal cortices was ten male and nine female dogs weighing from 15–20 kg. Nothing is known of these dogs except that they were adults in apparent good health during three weeks in our kennels prior to the beginning of the experiment.

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[1] Presented at the 65th Annual Meeting of the American Physiological Society.

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For the hypostimulated series, seven of the dogs were hypophysectomized by the temporal approach. The dogs tolerated the procedure well and began to eat on the third day. On the fifth day they were killed by an intravenous injection of chloroform; the adrenals were treated as in the previous experiment (Nichols and Little, 1951).

For the stress studies (hyperstimulated series), seven dogs were obtained after being used in other experiments being conducted in this laboratory. These dogs had been under barbiturate anesthesia for more than ten hours and had undergone considerable surgical manipulation, including cannulation of the femoral artery, repeated injection of epinephrine, mecholyl, regitine and various other vasodilator drugs. In most cases the dogs were in a state of profound hypotension. Eosinophil counts were zero in all cases. The dogs were killed with intravenous injection of chloroform and the adrenals treated as were the hypophysectomized ones.

For the cortical atrophy series, five dogs were given DDD 200 mg./kg. daily in capsules for two weeks. At the end of this time they were killed with chloroform and the adrenals were treated the same as those from the other dogs.

In all three series, immediately after the dogs were killed by injection of chloroform, the adrenals were removed to cold saline solution and the fat was trimmed away. Tangential slices were made as described in the previous paper, blotted dry, weighed on a microtorsion balance, and placed in the 10 ml. micro-respiratory chamber of the Warburg apparatus in which was 1.6 ml of Krebs-Henseleit solution. Half of the flasks had 5.0 mg. of ACTH[3] added to the menstruum. Incubation and readings were carried out exactly as in the previous paper.

RESULTS

Careful gross inspection of the skull and brain of all seven hypophysectomized dogs and sections of the brain at the site of hypophysectomy in five of the seven dogs failed to reveal anterior lobe tissue, suggesting that the hypophysectomy was complete.

Sections of the remaining unused portions of the adrenal cortex after the two slices had been cut from each side revealed that none of the medulla was included in any of the slices used for study. Figure 1 shows a wedge of a remnant from an unused portion of the adrenal of a dog five days posthypophysectomy. Figure 2 shows one of the first slices from the same adrenal gland after it had undergone two hours of incubation and shaking. This slice was cut in a template with a depth of 0.25 mm but micrometer measurements of the fixed and mounted specimen indicate the thickness to be 0.28 mm. This slice is seen to consist of a strip of capsule, zona glomerulosa and a few cells from the zona fasciculata. Figure 3 shows the second slice from the same gland; it consists almost exclusively of zona fasciculata. Although it was cut in the same template as the previous one the micrometer indicates the thickness to be 0.24 mm. The thickness of these slices

[3] The ACTH used in this experiment was Armour preparation 146R-S3, having 3.65 times the potency of the La-1-A standard. The amount in the respiratory flask was proportionally reduced to be the equivalent of 5.0 mg. La-1-A standard. The ACTH was obtained through the courtesy of Dr. Irby Bunding.

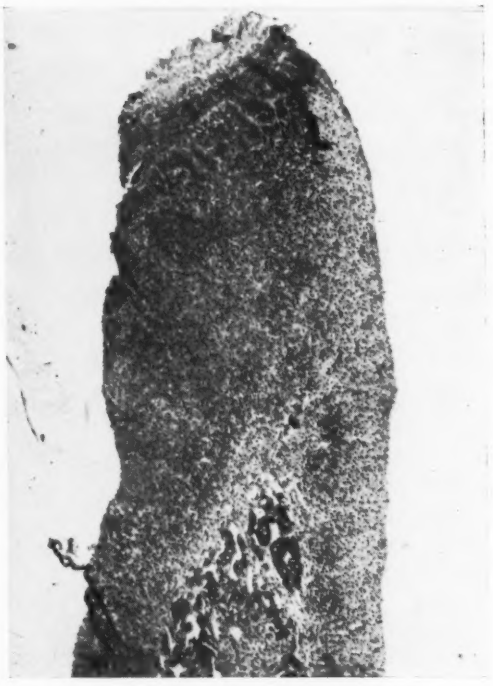

FIG. 1. Hematoxylin and Eosin ×45. This section shows the remaining unused portion of the gland after the four slices have been removed. Five days posthypophysectomy.

may have changed during the incubation and shaking. Although all three sections are from the gland of a dog five days posthypophysectomy no atrophic changes are apparent.

Sections from remnants and recovered slices of the adrenals of the surgically insulted dogs revealed the same microscopic picture as those from the hypophysectomized dogs.

The adrenals of the DDD treated dogs had undergone considerable atrophy and were too thin to cut separate slices of zona glomerulosa and zona fasciculata; therefore, only one slice was made. Recovered slices were found to consist of capsule, zona glomerulosa and portions of zona fasciculata undergoing atrophy. Figure 4 shows such a recovered slice. Micrometer readings indicate the thickness of these slices to be 0.33 mm. The slice pictured here is thicker than any of the others and was selected to show the extent of atrophy at this stage.

The oxygen consumption rates of the various slices with and without ACTH are shown in Table 1.

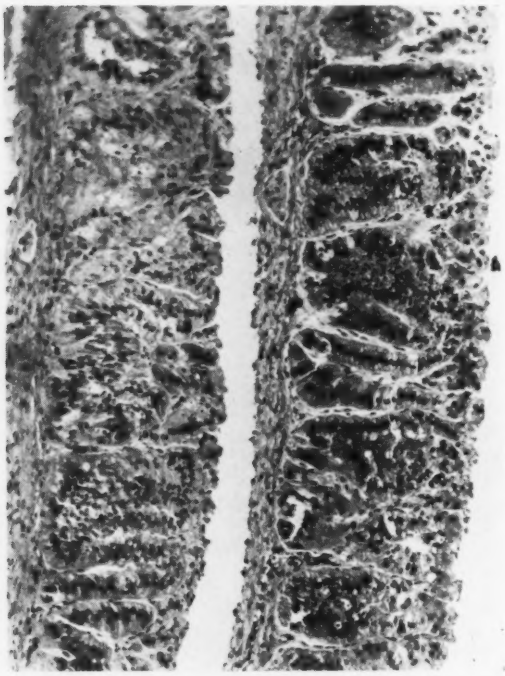

FIG. 2. Hematoxylin and Eosin ×120. This section shows two first slices tangentially cut from the same gland as shown in Fig. 1. These slices were recovered from the respiratory flask after two hours of incubation and shaking. Note that there is present a strip of capsule in addition to the zona glomerulosa and few cells of zona fasciculata.

TABLE 1

	1st slice "Glomerulosa and Fasciculata"						2nd slice "Fasciculata and Reticularis"					
	Without ACTH			With ACTH			Without ACTH			With ACTH		
	Mean	S.e.[1]	No.[2]	Mean	S.e.	No.	Mean	S.e.	No.	Mean	S.e.	No.
Normal dogs[3]	84.4[4]	±2.6	17	103.5[4]	±4.2	18	65.8[4]	±2.8	16	78.8[4]	±4.3	15
Dogs 5 days post-hypophysectomy	87	3.3	14	101	3.4	14	70	3.3	14	85	3.3	14
Dogs after surgical trauma	85	3	14	104	3.3	14	73	2.5	14	84	3.3	14
Dogs treated with DDD for two weeks	82	6	8	85	5.5	8						

[1] S.e. $= \sqrt{\frac{\Sigma d^2}{N(N-1)}}$ where d = difference between each observation and the mean of the observations and N = the number of observations.

[2] No. = Number of observations. In general two observations were made per animal.

[3] Nichols and Little, 1951.

[4] Oxygen consumption in mm.^{3}/100 mg./hr. of adrenal tissue as indicatedi n appropriate column.

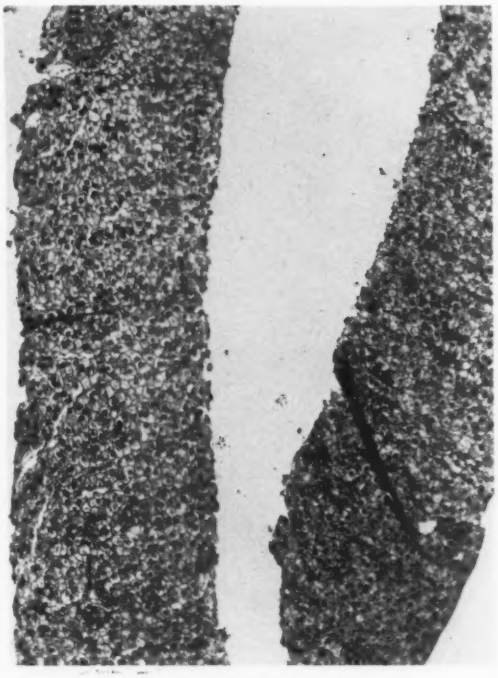

FIG. 3. Hematoxylin and Eosin ×120. This section shows two second slices from the same gland as shown in Fig. 1. These slices consist of predominantly zona fasciculata.

It can be seen that the oxygen consumption of the various zones did not differ significantly in any of the three groups and that they were not significantly different from the previously reported normal dogs. The oxygen consumption response of the various zones to ACTH in the hypostimulated, the stress and the previously reported normals, did not differ significantly; in the case of the dogs treated with DDD it is noted that while the oxygen consumption rate is unchanged, the addition of ACTH does not evoke an increase.

DISCUSSION

Injected ACTH is reported to have a short life in the animal body (Sayers *et al.* 1948; Richards *et al.*, 1950; Greenspan *et al.*, 1950; (van Dyke *et al.*, 1950; Richards and Sayers, 1951), being either inactivated or excreted within three hours. It is reasonable to assume, therefore, that the endogenous ACTH has disappeared from the hypophysectomized dogs and had been absent for about five days. Although a much longer time is re-

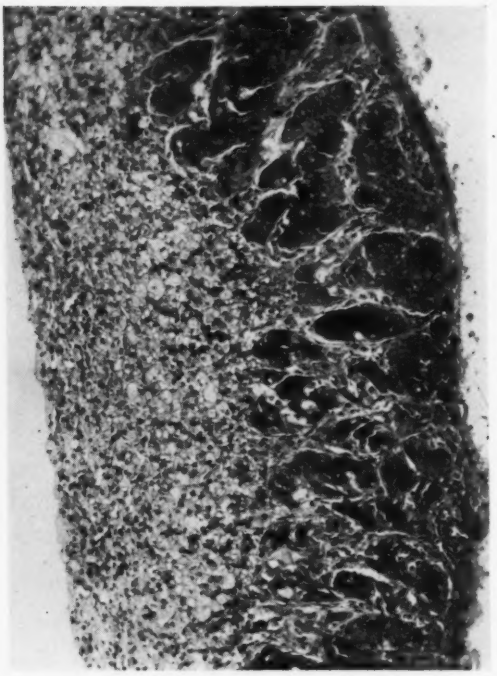

FIG. 4. Hematoxylin and Eosin ×120. This section shows a thicker than usual slice obtained from the adrenal of a dog which had been given DDD for two weeks. Note the disorganized pattern of zona glomerulosa but still apparently healthy in comparison to the deeper zona fasciculata undergoing atrophy and fatty degeneration.

quired for atrophy one might suspect that the tissues, after five days absence of ACTH, would have an altered rate of oxygen consumption and have become either sensitized or refractory to subsequent administered ACTH. In view of the fact that most authors report that hypophysectomy causes an atrophy of the zona fasciculata and reticularis with the zona glomerulosa remaining intact (see Nichols and Gardner, 1950 for bibliography) it was expected that most changes would take place in the second slice (zona fasciculata). However this was not the case. It should be noted that a recent paper by Lane and Debodo (1952) reports that significant atrophy occurs also in the zona glomerulosa after hypophysectomy. In the same line of reasoning one might have suspected that surgical trauma, which has amply been demonstrated to release ACTH as indicated by eosinopenia, would have stimulated maximally the adrenal gland and it would become refractory to subsequent ACTH. Again, this was not the case. It is interesting to note that Ferstl *et al.* (1951) report that the adrenal

cortex responds by an increase in oxygen consumption to substances other than ACTH, e.g., desoxycorticosterone. This finding was not confirmed by Overbeek and Van der Vies (1952) although the latter authors find crude low potency ACTH to be equally effective as highly refined high potency ACTH in stimulating an increase in oxygen consumption. They conclude that increase in oxygen consumption by adrenal cortical tissue is not specific for ACTH.

The finding that the rate of oxygen consumption by adrenal cortical slices undergoing atrophy from DDD is normal but does not increase when ACTH is added is not surprising when one considers the degree of atrophy present in these glands as indicated in Figure 4. Details of this atrophy have been discussed elsewhere (Nelson and Woodard, 1949; Nichols and Gardner, 1950; Nichols and Sheehan, 1953). There are other indications that such glands do not respond properly when ACTH is administered. Thus, dogs with atrophic glands exhibit a degree of insulin sensitivity (Nichols and Gardner, 1950), do not develop alloxan diabetes (Nichols and Sheehan, 1953), and on repeated administration of ACTH do not show diabetic glucose tolerance curves, do not develop an eosinopenia, do not show increased excretion of allantoin or uric acid, and do not give a diuresis (Nichols and Green, in press). All of these phenomena are observed when ACTH is given to a dog with intact adrenals.

The mechanism of this atrophy is unknown, but it has been suggested (Nichols and Gardner, 1950) that DDD may block the trophic action of ACTH. The findings reported here do not conflict with this hypothesis. DDD is a fat soluble substance and accumulates preferentially in the various fat depots of the body including the adrenal (Finnegan *et al.*, 1949). In unpublished experiments in which the ACTH was dissolved in a menstruum saturated with DDD the ACTH still caused an increase in oxygen consumption. (DDD is soluble in distilled water only to the extent of 1.4 mg./100 ml.) Apparently the DDD must be present in adrenal lipoids before this blocking effect is obtained, at least in the large concentration of ACTH used in these experiments. The action of DDD is further complicated by the fact that the dog is the only animal which suffers atrophy of the gland; the compound does not cause atrophy in the other usual laboratory animals (Nelson and Woodard, 1949), or in man (Sheehan *et. al.*, 1953). However Brown (1953) reports that rats do not respond to ACTH when pretreated with DDD despite the fact that atrophy of the gland has not taken place.

CONCLUSIONS

The rate of oxygen consumption per 100 milligrams of the hypostimulated zona glomerulosa and zona fasciculata of the adrenal cortices from

dogs five days posthypophysectomy and the hyperstimulated glands from surgically traumatized (stressed) dogs was normal and gave the usual increase in rate of oxygen consumption when ACTH was added *in vitro*. The rate of oxygen consumption of the atrophying tissue of the adrenal gland from a dog under the influence of DDD was normal but did not increase in the usual manner when ACTH was added. Three possible interpretations of these results may be made: (a) that none of the zones of the adrenal after five days without stimulus of endogenous ACTH have become either sensitized or resistant to subsequent ACTH as measured by oxygen consumption *in vitro*, (b) that adrenal tissue undergoing degeneration from DDD has a normal rate of oxygen consumption but does not react to administration of ACTH, and (c) adrenals which have been maximally stimulated by the animals own endogenous ACTH have a "normal" rate of oxygen consumption and still give an increase in rate when exogenous ACTH is added *in vitro*. This last point indicates that the conditions of the experiment are not physiological but are grossly pharmacological.

Acknowledgments

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EFFECTS OF GROWTH HORMONE IN DIABETIC DOGS[1]

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WHEN given to intact cats and dogs under appropriate experimental conditions highly purified growth hormone may produce diabetes (Cotes, Reid and Young, 1949; Campbell, Davidson, Snair and Lei, 1950; Campbell, Hausler, Munroe and Davidson, 1953). Permanent pituitary, or metahypophyseal diabetes has been produced in dogs by giving the injections for a longer period (Campbell, Davidson and Lei, 1950; Campbell, Chaikof and Davidson, to be published). The beta cells of the pancreatic islets exhibit extensive atrophic changes in these animals and only small amounts of insulin can be obtained from the pancreas. Milman and Russell (1950) found that growth hormone caused hypoglycaemia in normal fasting rats, but further elevation of the blood sugar in alloxan-diabetic rats. In depancreatized dogs Kurtz, de Bodo, Kiang and Ancowitz (1951) found that growth hormone given intravenously immediately after operation caused hypoglycaemia.

In the present experiments it has been found that the administration of purified growth hormone to diabetic dogs that were given their usual dose of insulin greatly intensifies the severity of the diabetes. Since these results indicated that the growth hormone had caused an increased demand for insulin by the extrapancreatic tissues, the effects of withdrawal of insulin from depancreatized dogs were also studied.

METHODS

The methods have been described by Campbell, Hausler, Munroe and Davidson (1953).

EXPERIMENTAL RESULTS

Effects of growth hormone on diabetic dogs

The purpose of this experiment was to determine the effects of purified growth hormone on 3 depancreatized dogs (D4, D5 and D6) and on a metahypophyseal diabetic dog (J). The depancreatized animals in metab-

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olism cages were given a diet of 400 gm. chopped horse meat, 200 gm. raw pancreas and 40 gm. sucrose daily, as two meals. The diabetes was controlled by 2 injections of insulin-zinc (Connaught Medical Research Laboratories), while dog D6 received in addition protamine zinc insulin in the mornings. These dogs were kept in this way for over 9 months. The metahypophyseal diabetic dog had been made permanently diabetic by the administration of purified growth hormone (Campbell, Lei and Davidson, 1951) and had remained in this state for 17 months. This dog received 2 injections of insulin-zinc and a morning injection of protamine zinc insulin, with the same diet as was given to the depancreatized dogs. The dogs appeared to be continuously in good health. The food was eaten as soon as it was given. Ketones (qualitative test) were absent from the urine and there was no visible lipemia.

These diabetic dogs were then given growth hormone, D21P, subcutaneously twice daily while the same dosage of insulin was maintained. (As described by Campbell, Hausler, Munroe and Davidson, this highly puri-

TABLE 1. DIABETIC DOGS GIVEN GROWTH HORMONE

The depancreatized dogs, D4, D5 and D6, and the metahypophyseal diabetic dog J were maintained on insulin and a diet containing raw pancreas during the preliminary or control period. The growth hormone D21P was injected subcutaneously in the dosage of 0.5 mg. per kg. of body weight on the first day, and 0.25 mg. per kg. per day during the next 3 to 4 days, together with the usual supply of insulin.

Diabetic dog no.	Body weight, kg.	Insulin, units per day		Urine, average values per day					
				Volume, ml.		Sugar, gm.		Nitrogen, gm.	
		I.Z.	P.Z.I.	Control	With G.H.	Control	With G.H.	Control	With G.H.
D4	15.6	44		550	1600	9.7	47.1	10.0	9.2
D5	19.7	36		450	1700	4.2	40.8	15.3	10.4
D6	10.1	8	6	280	1390	2.1	28.4	7.5	7.5
J	10.1	12	4	660	1250	13.1	27.1	10.6	9.7

fied growth preparation, D21P, previously produced diabetes in intact dogs in the dosage of 3.5 mg./kg. of body weight per day and, after a resting period of 30 days, again produced diabetes in 2 of these dogs when given in the dose of 2 mg./kg./day.) On the first day the diabetic dogs received 0.5 mg. of growth hormone D21P per kg. Due to the severity of the effects produced, the dosage was reduced to 0.25 mg./kg./day thereafter. These injections increased the urine volume and the sugar excretion (Table 1). The excretion of nitrogen, however, did not alter significantly during the short period of observation. These determinations are, however, of limited value due to failure to consume the full diet in some instances and to vomiting. On the day of the first injection polydypsia was noted, the urine volume and sugar content were above the previous values and ketones

were excreted. Two of the depancreatized dogs panted heavily and continuously in a manner similar to that previously found in intact dogs given larger doses of the growth hormone, while dog J did not eat all his food. On the second day of injection of growth hormone the dogs were depressed, even lethargic, and the panting continued. Polydypsia was marked and the urine tested strongly for ketones. The food was not readily eaten. The dogs D4 and J were most affected, they vomited and the former exhibited signs of dehydration. On the third day these signs were present in even greater degree. The blood plasma was visibly lipemic in all the dogs, being most evident in D4, while J, D5 and D6 followed in this order. The lethargy was marked except for D6. The dogs D4 and J refused food. On the fourth day of injection of growth hormone the dog D4 passed into coma, apparent-

TABLE 2. DEPANCREATIZED DOG D4, GIVEN GROWTH HORMONE

This depancreatized dog, D4, received growth hormone D21P, 0.25 to 0.5 mg. per kg. of body weight per day, with a constant dose of insulin.

Analyses, units	Before injections (average of 5 determinations)	Growth hormone injected		
		Values at end of:		
		Day 1	Day 2	Day 3
Urine				
Volume, ml.	150–1000	3200	1450	800
Sugar, gm.	3–15	25	96	20
Total N., gm.	4–24	5	21	7
Blood				
Sugar, mg. %	108–375	500	557	560
ESR, mm. in 60 min.	15–34	18	23	9
Red cells, vol. %	43–46	47	35	31
Hemoglobin, gm. %	11–13	14	12	10
Red cell count, 10^6 per cu. mm.	5.8–6.4	6.6	4.3	4.1
Clotting time, sec.	175–200	170	185	
Plasma				
Proteins, total, gm. %	5.75–6.26	6.58	4.90	4.48
albumin, gm. %	2.52–2.93	3.01	1.66	1.12
globulin, gm. %	2.51–3.11	3.07	2.83	3.19
fibrinogen gm. %	0.31–0.40	0.50	0.41	0.17
NPN, mg. %	35–39	57	95	169
Prothrombin time, sec.	12	12	12	

ly caused by diabetic acidosis, and expired about noon. The dog J was severely affected by the diabetes, as evidenced by dehydration, panting, polyuria, polydypsia, vomiting and ketosis. Despite grave doubts, the usual injections of growth hormone were given, but this proved fatal. The dog died during the following night and autopsy was performed on the fifth day. The dogs D5 and D6 were apparently in relatively good condition and were sacrificed on the 6th day.

Since it appears that the growth hormone produced kidney damage in dog D4, the data on this case are presented separately in Table 2. The

plasma non-protein nitrogen (NPN) increased in this dog to 4.5 times the initial value. The total protein of the plasma decreased on the second and third days due to decreased amounts of albumin and a terminal fall in fibrinogen. The kidneys were soft, and on cutting, a serum-like fluid exuded. Frozen sections revealed abundant amounts of stainable fat, for the most part in the distal convoluted and the straight segments of the tubules. Smaller amounts of fat appeared as fine droplets in the cells of the glomeruli. The haematocrit readings, red cell counts and haemoglobin concentrations of the blood decreased in the last 2 days.

In the other diabetic dogs given a constant dose of insulin the haematocrit readings, red cell counts and haemoglobin concentrations decreased slightly but not definitely on giving growth hormone. The total protein, albumin and globulin of the blood plasma did not obviously change during the relatively brief period of observation. The plasma NPN increased in dog J from 40 to 50 mg. % in the control period to 59 mg. % on the third day of injection, but did not change in the other two dogs.

During the administration of growth hormone the erythrocyte sedimentation rate (ESR) of the blood, and the fibrinogen concentration of the plasma increased in dogs D5, D6 and J (Fig. 1). In all the dogs a marked rise in total leucocytes occurred on giving growth hormone (Fig. 2). This was due largely to increase in the polymorphonuclear neutrophil cells. The stab, or band cells also increased in number. A slight apparent increase in monocytes, not sufficient to be definite, was noted. The percentages of the other types of leucocytes (per 100 cells) decreased, but the actual number of lymphocytes, eosinophils and basophils showed little change per cu. mm. of blood.

In 3 control, depancreatized dogs maintained on a steady dosage of insulin and given bovine plasma albumin instead of growth hormone these changes were not found.

In these diabetic dogs given growth hormone the weight of the liver, relative to body weight, was more than double that of normal dogs. The livers were friable and appeared grossly fatty. The dry, fat-free solids, which chiefly represent protein, were above normal. The total lipid contents of these livers were very great (Table 3). The kidneys were also enlarged and fatty. The dry fat-free solids of the kidney were above normal, and the total lipid contents were more than double the normal in 3 cases. In the other case (D4) the presence of much fat was demonstrated histologically.

In dog J the heart muscle was soft and had a yellowish cast. The histological observations, to be presented subsequently, showed an extreme degree of fatty infiltration in the muscle and a great quantity of total lipid

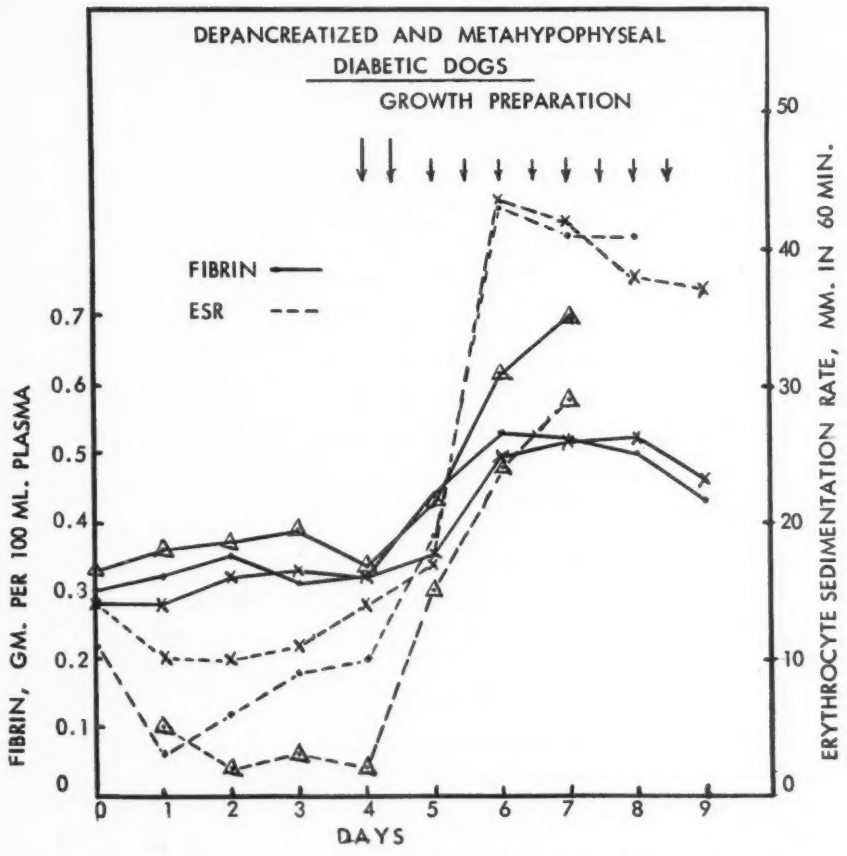

FIG. 1. Depancreatized dogs (D5, •; D6, x) and a metahypophyseal diabetic dog (J, △) given growth hormone preparation D21P in dosage of 0.5 mg. per kg. per day on day 4 and one-half this dose thereafter. The insulin dosage was unaltered.

was extracted (Table 3). In the other dogs the lipid content of the myocardium appeared to be slightly above normal.

Deprivation of insulin

Prior to the period of insulin deprivation the three depancreatized dogs were maintained on the diet containing raw pancreas and insulin-zinc in the dosage of 24, 28 and 24 units per day, as two injections, to dogs D1, D2 and D3 respectively. They had recovered from the operations which were performed 18 months previously in D1, and 1 month previously

TABLE 3. ORGAN WEIGHTS AND LIPID CONTENTS

The depancreatized dogs D4, D5 and D6, and the metahypophyseal diabetic dog J received injections of growth hormone with the usual dose of insulin as described in Table 1. The insulin dosage of the depancreatized dogs D1, D2 and D3 was reduced by one-half for 2 days, by one-half again for 2 days and was then withdrawn for 4 days from D1, and for 2 days from D2 and D3.

Diabetic dog no.	Treatment	Liver			Kidney			Heart	
		Weight	Dry, fat-free solids	Total lipid	Weight	Dry, fat-free solids	Total lipid	Dry, fat-free solids	Total lipid
		gm. per kg. of body weight						gm. per 100 gm. of fresh tissue	
D4	G.H. and insulin.	80.0	13.3	11.2	7.20	0.93		17.0	4.9
D5	G.H. and insulin.	48.2	7.4	15.6	7.25		0.70	16.6	5.8
D6	G.H. and insulin.	51.1	7.9	14.0	7.77	1.15	0.58	16.6	5.4
J	G.H. and insulin.	59.5	7.4	21.3	7.87	1.05	0.77	14.5	20.0
D1	Insulin withdrawn	44.1	5.8	15.4	6.59	1.11	0.60	19.5	7.0
D2	Insulin withdrawn	54.5	8.4	17.9	9.13	1.64	0.71	19.7	8.0
D3	Insulin withdrawn	72.5	9.5	29.4	6.58	1.28	0.50	18.0	6.4
Intact dogs									
Range, from		23.9	5.4	1.3	4.91	0.81	0.15	18.6	3.4
to		26.6	7.1	1.7	5.30	0.99	0.38	21.8	5.0
Average of 5		25.4	6.1	1.5	4.94	0.88	0.25	20.1	4.1

in the other dogs. The insulin dosage was reduced by one-half for 2 days. was again halved for the next 2 days and was then withdrawn completely. The dog D1 was sacrificed after 4 days, and the others after 2 days without insulin.

On withdrawal of insulin the dogs became depressed, the appetite waned and eventually signs of dehydration were evident. In the control period the urine volumes were about 300 ml. per day, and the sugar excretions ranged from 6 to 14 gms. of sugar. On withdrawal of insulin these rose to about 1400 ml. urine and 60 to 80 gms. sugar. Strongly positive tests for ketones occurred in the urine. The blood sugar levels, 16 hours after the last meal, ranged from 300 to 480 mg. % after removing insulin.

The ESR was high and variable (6 to 25 mm. per hour by the Wintrobe method) in dog D1, and did not increase significantly during insulin deprivation. In the other two dogs the ESR was initially in the normal range (about 1 and 5 mm. per hour in D2 and D3 respectively) and also did not change on withdrawing insulin. In dog D1 the plasma fibrin was initially rather high (0.32 to 0.35%) but was not altered by removal of insulin. The other plasma proteins were not noticeably altered. The haematocrit

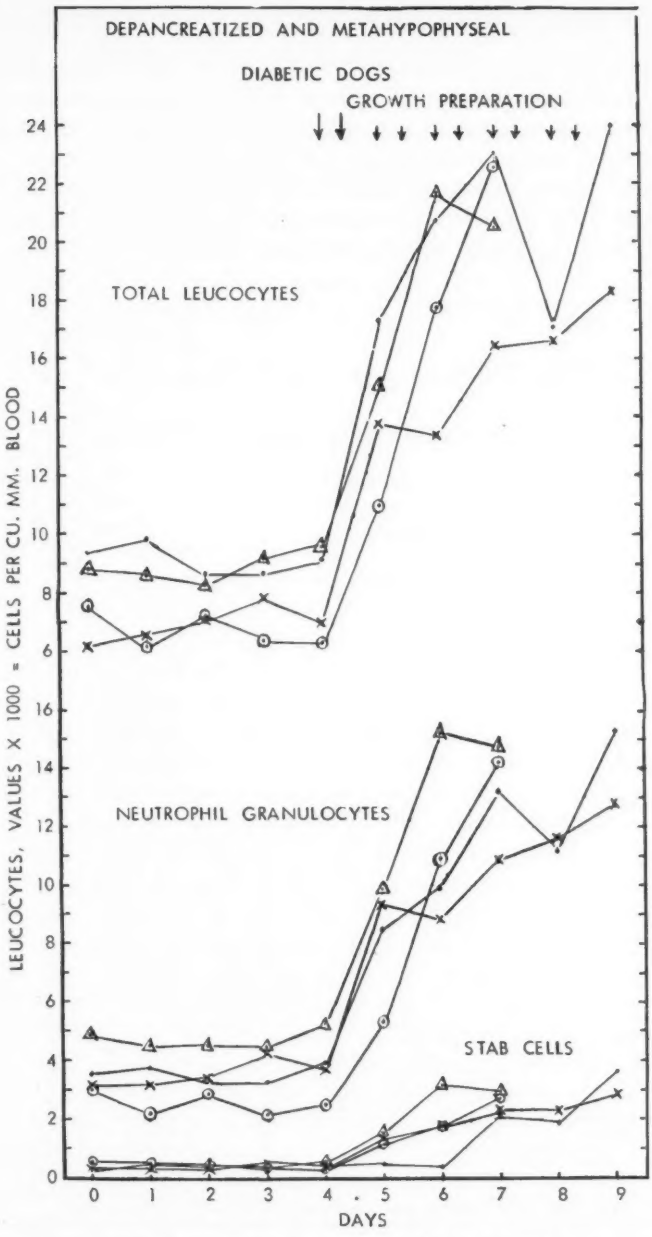

FIG. 2. Depancreatized dogs (D4, ⊙; D5, ●; D6, x) and a metahypophyseal diabetic dog (J, △) given growth hormone preparation D21P, in dosage of 0.5 mg. per kg. per day on day 4 and one-half this dose thereafter. The insulin dosage was unaltered.

readings and haemoglobin concentrations increased latterly, presumably due to dehydration. The clotting time of the blood and the prothrombin time of the plasma did not change noticeably. The blood NPN was initially 37 to 44 mg. % and rose to 61 mg. % on removing insulin.

The total leucocyte count of the blood rose during the period of insulin deprivation (Fig. 3). The leucocytosis was more pronounced in the dogs with the shorter, than in the dog with the longer postoperative interval.

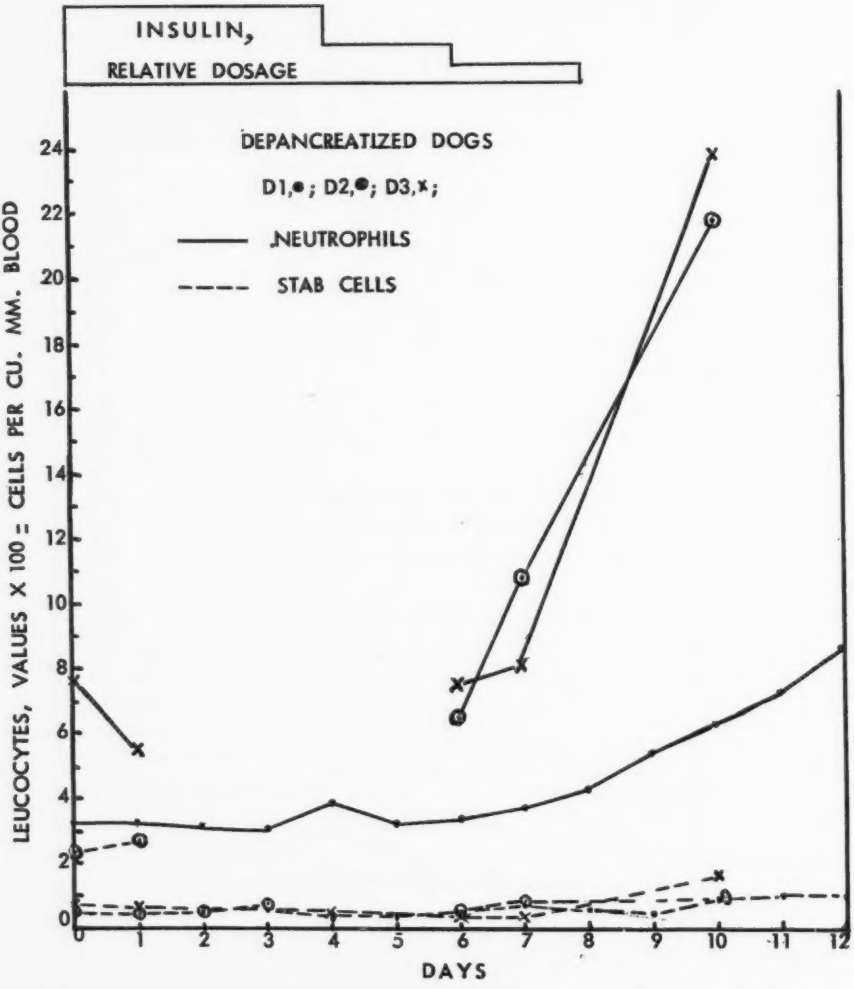

FIG. 3. Depancreatized dogs with insulin dosage reduced, then withdrawn completely.

This leucocytosis was due chiefly to increased numbers of polymorphonuclear neutrophil cells. The band cells did not increase (Fig. 3) to the same extent as in the diabetic dogs given growth hormone with the usual dose of insulin (per increase of 1000 neutrophils, the band cells increased in the former by 3 to 100, while in the latter by 200 to 290). The eosinophil count did not change appreciably.

The livers were enlarged, friable and very fatty at the end of the period of insulin deprivation. The liver weight and dry, fat-free solids were above normal in relation to body weight. The total lipids exceed the normal average by more than 10-fold (Table 3).

The weight, dry fat-free solids and total lipids of the kidneys were also above normal in the dogs deprived of insulin (Table 3). The hearts felt soft and had a yellowish cast. The fat content of the myocardium was above normal.

DISCUSSION

These observations show that the purified growth hormone intensified the diabetes of the diabetic dogs that were given their usual dose of insulin to such an extent as to cause fatal diabetic crises in the course of a few days. Recent evidence, derived from experiments on isolated tissues, indicates that this action of growth hormone could possibly be attributed either to interference with the uptake of insulin by the tissues, or with the action of insulin in the tissues (Stadie, Haugaard, Hills and Marsh, 1949). There are, however, several observations which render such explanations of the results not entirely adequate. Houssay, Biasotti and Rietti (1932) found that diabetogenic anterior pituitary extracts profoundly increased the severity of diabetes in depancreatized dogs. They found also that while hypophysectomized-depancreatized dogs could survive without insulin, the administration of diabetogenic extracts rapidly induced severe, and often fatal, diabetes. In the Houssay dogs, the factor of possible interference with the uptake or action of insulin is of course eliminated, and in them the effects of the diabetogenic extracts resemble the effects of growth hormone in our diabetic dogs. On withdrawal of insulin, depancreatized dogs survived for a comparable period in better condition than the diabetic dogs given growth hormone plus the usual dose of insulin. The growth hormone appears to have an effect greater than can be attributed to elimination of insulin action.

Campbell, Keenan and Best (1938) demonstrated that a diabetogenic anterior pituitary fraction (APE) increased the requirement for insulin in a depancreatized and a metahypophyseal (permanent pituitary) diabetic dog, since the doses of insulin needed to control the diabetes were increased by 3.3 and 8.8-fold respectively by the injection of APE. This partially-

purified globulin extract contained about 50% of growth hormone (Campbell, Davidson, Snair and Lei, 1950). Young (1944) showed that when diabetic or pre-diabetic dogs were given diabetogenic extracts of the anterior pituitary gland, the amounts of insulin required to control the diabetes were very great, and further, that the insulin given caused resumption of growth. These results indicate that the diabetogenic effects of the growth hormone cannot be satisfactorily explained as due only to elimination of the action of insulin. It is suggested that the growth hormone creates a greatly increased demand for insulin by the extrapancreatic tissues. This increased demand is obviously not exerted through the intervention of the pancreas, but as is shown in the following, may have a profound effect on the pancreas "*in situ*."

It is reasonable to assume that the diabetogenic, extrapancreatic effects of the growth hormone must be exerted in the intact animal as well as in the depancreatized animal. Indeed, the same growth hormone preparation produced diabetes in intact dogs (Campbell, Hausler, Munroe and Davidson, 1953). However, the doses of the growth hormone required to produce diabetes in the previously intact dogs (2 to 3.5 mg./kg./day) were greater than those that aggravated the diabetes in the depancreatized dogs given their usual supply of insulin. The effects of the lower doses in the depancreatized dogs were also more severe. The presence of the intact pancreas therefore greatly enhanced the resistance of dogs to the diabetogenic effects of the growth hormone. The administration of the relatively low dose of growth hormone also greatly intensified the diabetes of the metahypophyseal diabetic dog, given the usual dose of insulin. In metahypophyseal diabetes the metabolic defects can chiefly be attributed to atrophy of the beta cells of the islets of Langerhans and failure of these cells to secrete insulin at an adequate rate (Marks and Young, 1939; Haist, Campbell and Best, 1940). Thus the atrophy of the beta cells and failure of their functional capacity to secrete insulin greatly decreased the ability of the metahypophyseal diabetic dog to counteract the diabetogenic effects of the growth hormone. It appears therefore that the resistance of intact dogs to the diabetogenic effects of the growth hormone can be attributed in large measure to the insulin secreting activity of the beta cells of the pancreatic islets. It is reasonable to conclude that the growth hormone may create an increased metabolic demand for insulin, and also may increase the rate of secretion of insulin from the pancreas in intact dogs. This postulated period of increased insulin secretion must, of course, precede the phase of reduced secretion on exhaustion of the pancreatic islet cells.

Haist, Campbell and Best (1940), Haist (1944), and others, advanced the hypothesis that metahypophyseal, or permanent pituitary, diabetes

was caused by exhaustion of the beta cells of the pancreatic islets through overwork. This is supported by the evidence that the administration of large amounts of insulin with the diabetogenic extract tends to protect the islets and lessen the reduction in the insulin extractable from the pancreas (Best, Campbell, Haist and Ham, 1942). It follows from this hypothesis that, at some period during the induction of metahypophyseal diabetes, the rate of secretion of insulin from the pancreas must be increased. Since metahypophyseal diabetes has been produced by purified growth hormone preparations, it appears from this evidence also that the growth hormone may, under appropriate conditions, increase the rate of secretion of insulin.

Relationships between insulin and growth hormone are revealed by observations on the liver. von Mering and Minkowski (1890) observed after total pancreatectomy in dogs a high degree of fatty infiltration in the liver. Banting, Best *et al.* (1922a, b) found that the administration of pancreatic extracts containing insulin could prevent this massive deposition of fat in the liver. In the present study, shortly after the withdrawal of the insulin supply from depancreatized dogs the livers were enlarged and very fatty. Although the abdominal organs were not examined prior to stopping the injections of insulin, it has been our experience that under these conditions of adequate insulin and a diet containing raw pancreas, the lipid content of the liver is nearly normal. It can be concluded that severe or total deficiency of insulin causes acute and massive deposition of fat in the livers of dogs, and that insulin prevents this occurrence.

In the depancreatized dogs given their usual dosage of insulin, after a few days of injection of growth hormone the livers were much enlarged and very fatty. This great deposition of fat in the liver can be attributed to the growth hormone administered. When intact dogs were given larger doses of the same preparation of growth hormone the liver fat increased, but not to the same extent as in the depancreatized dogs (Campbell, Hausler, Munroe and Davidson, 1953). The degree of fat deposition in these intact dogs appeared to be related to the intensity of the diabetes and to the reduction of the insulin content of the pancreas caused by the growth hormone. The presence of the pancreas therefore protected the intact dogs in some measure from the liver fat-increasing effects of the growth hormone. Relatively small doses of growth hormone produced rapid and massive infiltration of fat in the liver of the metahypophyseal diabetic dog (with beta cell lesion) given the usual dose of insulin. The resistance of the intact dogs to the liver fat-increasing effect of the growth hormone can therefore apparently be attributed to the presence of functionally active beta cells. Thus, these observations on the liver also indicate that the resistance of intact animals to the growth hormone may be

due to an increased rate of secretion of insulin from the pancreas in response to an increased demand for insulin by the tissues.

The observations on the kidneys of dogs given growth hormone also agree with this interpretation. In depancreatized dogs the withdrawal of the insulin supply caused enlargement of the kidneys, increased the non-fat solids, which represent chiefly protein, and increased the lipid content. Relatively small doses of growth hormone, given to the depancreatized dogs and to the metahypophyseal dog with the usual dosage of insulin, also produced these effects. Larger doses of growth hormone in intact dogs caused some enlargement of the kidneys but did not noticeably increase the non-fat solids nor the fat content of the organs, although some infiltration of stainable fat was found on histological observation (Campbell, Hausler, Munroe and Davidson). While further data are required, there is no doubt that the relatively large amounts of growth hormone caused less change in the kidneys of the intact dogs than in the diabetic dogs supplied with insulin. It is again consistent with these findings to postulate that the growth hormone created an increased demand for insulin by the tissues of the depancreatized and the metahypophyseal diabetic dogs, and in the intact dogs caused an increased rate of secretion of insulin from the pancreas.

From studies of the effects of growth hormone in normal and diabetic rats, Milman and Russell (1950) favored the explanation that the hormone increased the rate of secretion of insulin from the pancreas. Milman, DeMoor and Lukens (1951) also suggest, from studies of nitrogen metabolism in cats, that growth hormone may increase insulin secretion. It should also be noted that immediately after depancreatectomy in dogs, the administration of growth hormone causes hypoglycemia (Kurtz, de Bodo, Kiang and Ancowitz, 1951).

On the other hand, Anderson and Long (1947) found that the addition of growth hormone to the fluid perfusing the pancreas of the rat reduced the secretion of insulin that was elicited in response to glucose. This result indicates that growth hormone may directly inhibit the secretion of insulin from the pancreas that is isolated from the other tissues of the body. The possibility arises therefore that there may be two opposing effects of growth hormone on the pancreas—a direct inhibitory effect and an indirect effect exerted through the extrapancreatic tissues and causing stimulation of insulin production. It would appear that, under the conditions described above, the indirect stimulating effect of growth hormone on the secretion of insulin may be the more powerful and may overcome the inhibitory effect.

According to Allen (1927), Joslin, Root and White (1927) and Heck and Hall (1939) leucocytosis is found in patients in diabetic acidosis and in

coma. On restoring control of the diabetes by insulin, etc., the leucocyte counts returned to normal. This leucocytosis has been ascribed to acidosis (Allen) and to hypertonicity of the blood plasma (Tullis, 1948), but the cause has apparently not been definitely established. On reduction, and then withdrawal of the insulin supply to the depancreatized dogs marked leucocytosis, due to neutrophilia, occurred. Ketosis, but not coma, appeared in these dogs. In depancreatized dogs given maintenance doses of insulin the leucocyte counts of the blood were normal, but on giving growth hormone neutrophilia occurred. In these diabetic dogs given growth hormone the number of band cells increased more than in the dogs deprived of insulin. In intact dogs it was found (Campbell, Hausler, Munroe and Davidson) that larger doses of growth hormone caused marked neutrophilia and also increased the number of band cells. It appears unlikely that the increase in neutrophils in these intact dogs can be ascribed solely to relative deficiency of insulin, since these cells were definitely increased on the day following the first injection and since the band cells were more numerous than in the diabetic dogs deprived of insulin. The neutrophilia elicited in normal dogs by growth hormone cannot be ascribed to hyperglycemia nor to ketonuria, since it occurred in a dog in which these signs were absent. Examination of smears of the bone marrow have not so far revealed significant changes in the frequency distributions of the various cell types in the dogs given growth hormone, but studies on sections of the bone marrow are in progress.

In normal dogs, growth hormone elicits a prompt and progressive increase in the ESR of the blood and in the fibrinogen of the plasma (Campbell, Hausler, Munroe and Davidson). The increased rate of sedimentation in these circumstances can be chiefly attributed to the effect of fibrinogen in promoting aggregation of the blood cells. It appears probable also that the growth hormone accelerates the production of fibrinogen by the liver. Since the ESR and plasma fibrinogen did not change on withdrawal of insulin from the depancreatized dogs, it is apparent that the effects of growth hormone on ESR and on fibrinogen in the normal dogs cannot be attributed to deficiency of insulin, nor to the accompanying hyperglycemia and acidosis.

The administration of growth hormone to the depancreatized and the metahypophyseal diabetic dog given the usual amount of insulin increased plasma fibrinogen and the ESR, but to a lesser extent than in the normal dogs given growth hormone. It is possible that insulin may be necessary for the occurrence of these responses. Whether the lesser repsonses in the diabetic animals given insulin and growth hormone are due to the lesser dosage of the growth hormone or to lesser amounts of available insulin is not known.

The observations on the size and composition of the kidney and on its histological appearance (to be described subsequently) are of interest in relation to the signs of functional failure observed in two of the diabetic dogs given growth hormone. The fatty degeneration of the heart in the metahypophyseal diabetic dog given growth hormone, and the lesser changes in the myocardium in the depancreatized dogs deprived of insulin are also of interest.

SUMMARY

Injections of purified growth hormone greatly aggravated the severity of diabetes in depancreatized dogs and in a metahypophyseal diabetic dog that were maintained on a steady dose of insulin.

In these diabetic dogs given their usual supply of insulin the injections of growth hormone increased the plasma fibrin and the erythrocyte sedimentation rate (ESR). Leucocytosis due to marked increases in neutrophil granulocytes and lesser increases in band cells occurred. Massive deposition of fat occurred in the liver and the content of fat-free solids increased. The lipid content and the fat-free solids of the kidney also increased. Severe fatty degeneration of the heart occurred in the metahypophyseal diabetic dog given growth hormone.

Evidence from depancreatized dogs deprived of insulin indicates that the increase in liver and kidney fat of the dogs given growth hormone may be due, in part at least, to a relative deficiency of insulin. Withdrawal of insulin from depancreatized dogs was followed by neutrophilia, with a relatively small effect on stab cells. In these dogs the plasma fibrin levels and the ESR were high and rather variable but were not altered significantly by lack of insulin.

Evidence is given which suggests that the growth hormone creates an increased metabolic demand for insulin, and increases, at an early stage of its effects, the rate of secretion of insulin by the pancreas.

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MECHANISMS OF INSULIN AND EPINEPHRINE EFFECT ON THE LEVEL OF PLASMA POTASSIUM

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IT HAS been shown that the potassium content of plasma, liver, and muscle are altered following the administration of insulin or epinephrine in the rat (Dury, 1951; Dury and Johnston, 1951; Dury and Moss, 1952). Further studies of the influence of these hormones on potassium mobilization showed that insulin and epinephrine moderated an induced plasma potassium plethora following an infusion of glucose in adrenalectomized-alloxanized rats. Although both hormones induce a significant decrease in the plasma potassium level of intact rats, insulin and epinephrine have divergent effects on the potassium content of plasma and other tissues in adrenalectomized, adrenal-enucleated, and adrenalectomized-alloxanized rats. The results of these investigations indicated that insulin induced a fall in plasma potassium level in consequence of its effect upon carbohydrate assimilation and utilization when carbohydrate reserves are adequate or glucose is made available by exogenous supply (Dury, 1951; Dury and Moss, 1952; Dury, 1952; Treadwell and Dury, 1953). The manner of epinephrine's effect on tissue potassium content was not apparent from the data of these experiments. The divergent effects of the two hormones on the potassium content of tissues following their administration in the different types of animals used therefore suggested that they reflected differences in the action of epinephrine and insulin on intermediary metabolism.

In attempting to elucidate the mechanisms of action of insulin and epinephrine on the level of plasma potassium and their divergent effects on the potassium content of other tissues the experiments reported here were undertaken to observe concurrently the effects of these two hormones on liver electrolytes, glycogen and lipid partition, and plasma constituents. For reasons discussed in detail in a previous paper (Dury, 1952) the alloxanized-adrenalectomized type of animal was selected as the one of choice for these experiments. In order to avoid the vitiating effects on intermediary metabolism which may occur consequent to the absence of the permissive or "conditioning" influences (Ingle, 1951) of adrenocortical hormones following adrenal gland ablation, these animals were injected with small maintaining levels of Lipo-adrenal extract.

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MATERIALS AND METHODS

Male Wistar rats weighing 250–300 grams were used. Alloxan monohydrate (Eastman #1722) was prepared fresh for use as a 5% solution and injected directly into an exposed saphenous vein at a dose level of 40 mg./kgm. body weight. The rats were maintained on tap water and a laboratory chow biscuit *ad libitum* for the following 72 hours in individual cages. Those which developed a definite diuresis during this period were bilaterally adrenalectomized. They were given 0.3 ml. of Lipo-adrenal extract (Upjohn)[1] via subcutaneous injection immediately after ablation of the adrenal glands and injected with the same amount on each of the next two days. During this period following adrenalectomy a 1% NaCl solution for drinking purposes was substituted for the tap water. The rats were sacrificed on the morning of the third day after adrenalectomy (6 days after alloxan injection) *sans* overnight fast. All surgical procedures, injections of agents, and removal of tissues for analyses were done while the rats were lightly anesthetized with *EVIPAL* (n-methyl-cyclo-hexenyl-methyl barbituric acid).

Groups of alloxanized-adrenalectomized rats were prepared for use in the experiments as follows: *Controls*—not treated; *Insulin*—(*ILETIN*, regular, diluted fresh for use with physiological saline) injected subcutaneously at the dose level of 0.5 Units per rat 60 minutes before tissues were taken for analyses. *Epinephrine*—(Adrenalin tablets; Parke, Davis & Co.; prepared fresh for use in physiological saline) injected subcutaneously at the dose level of 0.04 mg./100 gm. body weight (approx. 0.2 ml.) 60 minutes before tissues were taken for analyses. *Glucose infusion*—2 ml. of a 50% solution freshly prepared in Sorensen's M/15 sodium phosphate buffer solution, pH 7.4 injected in the course of 3–4 minutes directly into an exposed saphenous vein. Tissues for analyses were taken 60 minutes after the glucose infusion. *Insulin pretreated-Glucose infusion*—0.5 Unit of insulin was given by subcutaneous injection 5 minutes *before* 2 ml. of the glucose infusion was administered; tissues were obtained 60 minutes after the infusion. *Epinephrine pretreated-Glucose infusion*—0.04 mg. epinephrine per 100 gm. weight was given by subcutaneous injection 5 minutes *before* 2 ml. of the glucose infusion was administered; tissues were taken for analyses 60 minutes after the glucose infusion.

At the designated time the animals were anesthetized and cardiac blood obtained in a syringe moistened with one drop of heparin (*LIQUAEMIN*, 10 mg./ml.; Eli Lily & Co.). The blood was immediately centrifuged and aliquots of plasma taken for the determination of sodium, potassium, chloride, glucose, and water content. The abdomen and chest were opened immediately after the cardiac blood was secured; the heart was incised and allowed to bleed. A specimen of liver for glycogen determination was taken, gently blotted on filter paper, and put in a tared flask charged with 30% KOH. Another specimen of liver (approx. 1 gm.) was taken for the determinations of water and electrolytes content. The remainder of the liver was dissected free of adherent tissues and taken for the determination of liver lipids (total and fractions). All tissue samples were weighed on a chainomatic balance immediately after removal from *situ*. The procedures for the determinations of plasma and liver water and electrolytes, and liver lipids content were the same as previously described in use in this laboratory. Water content of plasma and liver was calculated by difference from the wet weight after drying the specimens for 16 hours at 105 degrees C. The liver specimens were ashed overnight at 425 degrees C. and sodium and potassium content of liver tissue were determined on suitable aliquots of the ash with the aid of a duo-optical designed internal lithium standard flame pho-

[1] We wish to thank Dr. H. F. Hailman for the gift of the Lipo-Adrenal Cortex samples, Registered Trade-Mark, The Upjohn Company.

tometer. Plasma sodium and potassium content were determined directly after proper dilutions with the aid of the flame photometer. Plasma chloride was determined by the method of Schales and Schales (1941); plasma glucose by the method of Kingsley and Reinhold (1949); liver glycogen by the method of Good, Kramer, and Somogyi (1933) and glucose equivalents after acid hydrolysis by iodometric titration method of Somogyi (1945). The liver samples taken for lipids analyses were macerated in a mortar with sand and taken up in 95% ethyl alcohol. Extraction of the liver lipids was accomplished by refluxing 3 times with 95% alcohol and thrice with ether followed by distillation under reduced pressure of the combined extracts. The residue was taken up in petroleum ether (30–60 degrees); washed 3 times with an equal volume of water; then the petroleum ether extract was dried over anhydrous sodium sulfate and made up to a convenient volume. Suitable aliquots of the petroleum ether were taken for the determination of the lipid partition as follows: Total lipids was calculated from weight of residue after distillation of the petroleum ether and drying to constant weight; lipid phosphorous by a modification of the Youngsburgs' method and the method of Fiske and SubbaRow (1925); phospholipids (derived from the lipid phosphorous values $\times 26$); total and ester cholesterol by the method of Schoenheimer and Sperry (1934); neutral fat content was derived by calculation of difference.

RESULTS

The comparative effects of insulin and epinephrine on the content of the several fractions of liver lipids, liver glycogen, and the water and electrolytes content of liver and plasma 60 minutes after their administration in alloxanized-adrenalectomized rats (given maintaining doses of Lipoadrenal extract) are shown in Table 1. It is evident from the change in the plasma glucose level and liver glycogen content that carbohydrate oxidation was effectively influenced following the administration of insulin. A significant decrease in the liver content of the ester cholesterol content also was found. Particularly noteworthy in these results is the absence of any change in the potassium content of liver or plasma. In contrast to this is the marked fall in the plasma potassium level ("P" = <0.0001) and the lowered plasma sodium level found 60 minutes after the injection of epinephrine. The changes in liver glycogen content and plasma glucose level clearly show the classical influence of epinephrine effect on carbohydrate metabolism. Except for a significant rise in the free cholesterol and decrease in the ester cholesterol content of the liver there were no other changes in the content of liver constituents for which determinations were made.

The next step was to determine the effect of insulin and epinephrine on the potassium content of liver and plasma when an exogenous supply of glucose was made simultaneously available to this type of animal. The possibility that insulin induced a significant fall in plasma potassium level in association with an increase in carbohydrate assimilation and utilization which was indicated in previous studies with intact rats and the effectiveness of insulin to moderate plasma potassium plethora (Dury, 1951; Dury,

TABLE 1. EFFECT OF INSULIN AND EPINEPHRINE ON CONSTITUENTS OF LIVER AND PLASMA IN ALLOXANIZED-ADRENALECTOMIZED[1] RATS

Constituents	Not treated (8)	Insulin[2] (7)	Epinephrine[3] (8)
Part A. Liver lipids partition and glycogen content			
Total lipids gm. %	5.00 ± .14	5.06 ± .12	5.39 ± .12
Phospho-lipids gm. %	4.45 ± .25	4.50 ± .27	4.54 ± .02
Total cholest. gm. %	.23 ± .006	.22 ± .004	.23 ± .004
Free Cholest. gm. %	.19 ± .004	.20 ± .005	*.22 ± .003*†
Ester Cholest. gm. %	.04 ± .006	*.02 ± .003**	*.02 ± .003*†
Neutral Fat gm. %	.42 ± .13	.52 ± .18	.60 ± .12
Glycogen mg. %	2047 ± 116	*1412 ± 66*†	*535 ± 72*†
Part B. Liver and plasma water and electrolytes content			
Liver:[4]			
Total water gm.	712 ± 2.0	714 ± 2.0	707 ± 2.0
Potassium m.eq.	94.8 ± 1.2	97.7 ± 1.9	94.8 ± 1.9
Sodium m.eq.	28.7 ± 1.3	31.9 ± 2.1	28.6 ± 1.4
Plasma:[5]			
Water %	92.9 ± .1	92.8 ± .09	92.6 ± .17
Chloride m.eq.	112.5 ± 1.7	117.2 ± 1.8	111.3 ± 1.1
Potassium m.eq.	5.52 ± .16	5.06 ± .34	*3.78 ± .23*†
Sodium m.eq.	168.8 ± 1.3	168.5 ± 1.7	*163.7 ± .8*†
Glucose mg. %	230 ± 50	*72 ± 26*†	*383 ± 44**

Figure in parentheses is number of rats in group. All values are Mean ± S.E. Significant differences between means of control and respective experimental values are *italicized:*

* "P" = 0.05 to 0.01.

† "P" = 0.01 and lower.

[1] See text for schedule of injections of Lipo-adrenal extract.

[2] Insulin injected sub-Q at 0.5 Units/rat 60 minutes before tissues were obtained.

[3] Epinephrine injected sub-Q at 0.04 mg. per 100 gm. rat 60 minutes before tissues were obtained.

[4] Constituents are represented per Kgm. wet organ weight.

[5] Electrolytes are represented per Kgm. plasma water.

1952) was looked for. Inspection of Table 2 shows that insulin injection five minutes before infusion of 1 gm. of glucose in these rats induced a significant increase in liver potassium content ("P" = <0.0001) and a significant decrease in plasma potassium level ("P" = 0.001). It is also evident that there were several other significant changes in liver and plasma content of constituents with insulin pre-treatment 60 minutes after the glucose infusion. The plasma sodium level was significantly greater and the liver sodium content was lower than the values of the respective constituents in the controls. There also were found several significant changes in content of liver lipids: Total liver lipids, the phospholipids content, and total cholesterol content was significantly lower than the respective values in the controls; and the neutral fat content was greater than the control value. The plasma glucose level is clearly indicative (compared to the glycemic level in the controls) of a status of increased carbohydrate utilization.

TABLE 2. EFFECT OF PRE-TREATMENT WITH INSULIN OR EPINEPHRINE ON CONSTITUENTS OF LIVER AND PLASMA IN ALLOXANIZED-ADRENALECTOMIZED[1] RATS 5 MINUTES BEFORE INFUSION OF A GLUCOSE SOLUTION[2]

Constituents	Glucose infusion alone (9)	Insulin Pre-Rx plus glucose (6)	Epineph. Pre-Rx plus glucose (7)
Part A. Liver lipids partition and glycogen content			
Total lipids gm. %	4.81 ± .09	*4.43 ± .13**	4.97 ± .18
Phospho-lipids gm. %	4.26 ± .10	*3.49 ± .12†*	4.25 ± .15
Total cholest. gm. %	.22 ± .005	*.20 ± .005**	.22 ± .006
Free Cholest. gm. %	.20 ± .003	.19 ± .003	.20 ± .009
Ester Cholest. gm. %	.02 ± .002	.01 ± .005	.02 ± .003
Neutral Fat gm. %	.32 ± .08	*.72 ± .11†*	.49 ± .08
Glycogen mg. %	2091 ± 231	1871 ± 246	*808 ± 327†*
Part B. Liver and plasma water and electrolytes content			
Liver:			
Total water gm.	720 ± 2.0	723 ± 4.0	714 ± 1.0
Potassium m.eq.	89.8 ± 1.3	*102.5 ± .9†*	*95.2 ± 2.1**
Sodium m.eq.	33.7 ± 1.4	*28.1 ± 1.4**	31.5 ± 1.8
Plasma:			
Water %	93.3 ± .14	93.3 ± .05	92.6 ± .09
Chloride m.eq.	109.4 ± 2.6	112.2 ± 3.2	110.8 ± 1.2
Potassium m.eq.	4.67 ± .10	*3.33 ± .13†*	*3.59 ± .28†*
Sodium m.eq.	160.2 ± 1.5	*174.9 ± .6†*	163.8 ± 1.4
Glucose mg. %	500[3]	*347 ± 59†*	500[3]

Figure in parentheses is number of rats in group. All values are Mean ± S.E. Significant differences between means of control and respective experimental values are *italicized:*

* "P" = 0.05 to 0.01.

† "P" = 0.01 and lower.

[1] See text for schedule of injections of Lipo-adrenal extract.

[2] 2 ml. of 50% glucose solution given intravenously; tissues were obtained 60 minutes after the glucose infusion. See legends of Table 1 for dose levels of insulin and epinephrine used in pre-treatment 5 minutes before injection of glucose infusion.

[3] These glucose values are at least 500 mg. %.

Reference to Table 2 shows the results of liver and plasma constituents found 60 minutes after infusion of 1 gm. of glucose in rats which were pre-treated with epinephrine five minutes before the glucose infusion. In this group as in that administered epinephrine not followed by glucose (Table 1) there was found a significantly lowered plasma potassium level and a significantly increased liver potassium content. Insofar as the changes in these constituents were observed the results of insulin and epinephrine pre-treatment were qualitatively the same. It is evident from an inspection of the remainder of the results in this group that there were marked differences in the effect of insulin and epinephrine on liver and plasma constituents. It is especially noteworthy that epinephrine's influence on plasma and liver potassium change was found in association with clear evidence of an extant hyperglycemia and markedly lowered liver glycogen content evidently the consequence of epinephrine-induced liver glycogenolysis.

DISCUSSION

It is generally accepted that there exists a relationship between potassium and carbohydrate metabolism. Until recently, the evidence which appeared in the literature (see refs. quoted by Dury, 1951) suggested that the fall in plasma or serum potassium level after the injection of insulin or epinephrine were specific actions of both hormones, or the consequence of the influence of both hormones upon carbohydrate metabolism. Dury (1951), and Dury and Moss (1952) showed that in overnight fasted rats epinephrine induced a lowered plasma potassium which did not involve the mediation of the adrenal cortex; and that insulin's apparent effect on the plasma potassium level in intact rats was mediated via the stimulation of epinephrine from the adrenal medulla since insulin was ineffective in the adrenalectomized and the adrenal-enucleated rat. Further investigations of this problem clearly indicated that insulin's effect upon the level of plasma potassium was related to changes in carbohydrate metabolism consequent to the influence of the hormone on the latter (Dury, 1952; Treadwell and Dury, 1953). The manner in which epinephrine "effected" a fall in plasma potassium level could not be deduced from the available data.

Since the mechanism of action of insulin and epinephrine effect on plasma and tissue potassium content was unsettled, the experiments presented here were undertaken to observe the effects of the two hormones concurrently upon liver lipid partition, glycogen, electrolytes, and plasma constituents. The alloxanized-adrenalectomized rat was chosen for these studies to eliminate the possibilities of reflex stimulation and endogenous secretion of insulin, epinephrine, or adrenocortical hormones when either the insulin or epinephrine was administered (Dury and Moss, 1952; Somogyi, 1949; v. Euler and Luft, 1952). The evidence presented here in Tables 1 and 2 confirm those of previous investigations that insulin administration results in a lowered plasma potassium level only when certain conditions are extant; namely, a large carbohydrate reserve or exogenous supply is available. Furthermore, if the latter condition does not exist then insulin is also ineffective in inducing a change in the plasma potassium level in the absence of the endogenous source of epinephrine. The results presented in Table 2 indicate that the lowered plasma potassium content in the insulin pre-treated group occurred in conjunction with mobilization of potassium into the liver. That these changes were induced consequent to insulin action promoting carbohydrate utilization are indicated by the glycemic level found in these animals, the increased neutral fat fraction, and the decreased phospholipid and total cholesterol fractions. These

results conform with the findings of Kaplan and Chaikoff (1935) of a reduction in liver phospholipids in depancreatized dogs maintained with insulin; and the reports of Stettin and Boxer (1944), Stettin and Klein (1946), and Chernick and Chaikoff (1950) that hepatic lipogenesis is promoted in the alloxan diabetic rat given a high carbohydrate diet and injected with insulin. These changes are accepted as indicative of increased hepatic utilization of carbohydrate consequent to insulin action.

The mechanism of action of epinephrine on the plasma potassium level cannot be deduced from the information obtained in these experiments. It is abundantly clear from the data presented in Tables 1 and 2 that the lowered plasma potassium level after epinephrine administration cannot be attributed to an increased level of hepatic carbohydrate utilization. It is also evident that epinephrine induced a fall in plasma potassium level in the absence of adreno-cortical mediation; in the absence of possible insulin influences on the resultant final change found in potassium content; and the lack of influence of the relative status of carbohydrate metabolism of the animal. Some of these points were suggested in the results obtained in other studies reported from this laboratory on this problem. It is evident from these results that measurement of changes in hepatic constituents in conjunction with those in the plasma cannot provide the key to the mechanism of action of epinephrine on potassium mobilization and concentration in the different compartments of the body. Therefore, consideration should be given to the possibility that plasma potassium fall after epinephrine is the resultant of the latter affecting metabolism in the blood or peripheral tissue. This aspect of the problem is under investigation. The results of this and other related studies indicate however that epinephrine should be considered as a humoral agent having an integral part in the mechanism of potassium homeostasis.

SUMMARY

Alloxanized-adrenalectomized groups of rats given maintaining injecing injections of Lipo-adrenal extract were used in an attempt to elucidate the mechanism of actions of insulin and epinephrine on the level of plasma potassium. Determinations were made of the liver content of the several lipid fractions, liver glycogen and electrolytes content, and plasma glucose and electrolytes content in this type of animal 60 minutes after the injection of insulin or epinephrine, and in groups 60 minutes after the infusion of 2 ml. of 50% glucose solution which had been pre-treated with insulin or epinephrine.

The results showed that insulin administration induced a significant decrease in the level of plasma potassium only when a large exogenous supply of glucose was made available. It was shown that insulin adminis-

tration does not induce a lowered plasma potassium level in the alloxanized-adrenalectomized rat just as was demonstrated in previous studies from this laboratory that it was incapable of effecting this change in the adrenalectomized or the adrenal-enucleated rat. The results indicated that insulin's effect on plasma potassium was in association with carbohydrate utilization. Epinephrine induced a lowered plasma potassium level in this type of animal without glucose infusion as well as in the group given the glucose solution. These results conform with those of other studies in the intact, adrenalectomized, and adrenal-enucleated rat. The mechanism of action of epinephrine on the plasma potassium level could not be deduced, however, from the present data. The fact that epinephrine does induce this change in such a variety of conditions was considered evidence of it having an integral role in the mechanism of potassium homeostasis.

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EFFECT OF DIETHYLDITHIOCARBAMATE AND OTHER AGENTS ON MICE WITH THE OBESE-HYPERGLYCEMIC SYNDROME[1,2]

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ESSENTIAL characteristics of the obese hyperglycemic syndrome of mice have been described in previous reports (Mayer *et al.*, 1952, 1953; Bleisch, Mayer and Dickie, 1952; Guggenheim and Mayer, 1952, etc.). The present report deals essentially with the nature of the hyperglycemia and suggests a possible endocrine origin for the whole syndrome.

In early studies (e.g. Mayer, Bates and Dickie, 1951, Mayer, *et al.*, 1953) all animals manifesting the obese-hyperglycemic gene showed high blood glucose levels (always over 180 mg. % after 3 months of age, generally of the order of 300 mg. % after 4 months). Recently, with the accelerated production of these animals from a genetically heterogeneous stock, a certain proportion of obese animals between 3 and 8 months old were observed to have normal blood glucose levels associated with all usual characteristics of the obese hyperglycemic syndrome, including hypercholesterolemia. This suggested either that the diabetes occurred genetically independently of the obesity or, as was more likely, that a genetic factor (or genetic factors) had appeared in the stock which masked or greatly delayed the "diabetic" aspect. If this were the case, as extreme sensitivity to growth hormone has been observed to be a characteristic feature of the obese hyperglycemic factor (Mayer *et al.*, 1953, Mayer and Silides, 1953), while non-obese animals are unresponsive to even prolonged and massive treatment, it appeared probable that the hyperglycemia might still be elicited and the diabetic-like condition "unmasked" or accelerated by growth hormone. This was found to be the case in a series much larger than had hitherto been available.

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[2] This work was in part presented at the meeting of the Federation of Societies of Experimental Biology, Chicago (Illinois) April 10, 1953 (Fed. Proc. 12, 423, 1953).

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The role of the pancreas appears central to this syndrome. Histologically, this organ presents the only abnormalities of possible etiologic significance (Bleisch, Mayer and Dickie, 1952); the islets of Langerhans show a striking increase in both number and cellularity. The resistance to insulin and sensitivity to pancreatectomy (Mayer *et al.*, 1953) are also suggestive. It therefore appeared of interest to study the effect of administration of an agent reported to damage the alpha cells of the islets of Langerhans, diethyldithiocarbamate (Kadota and Midorikawa, 1951),[4] and of cobaltous chloride (Goldner, Volk and Lazarus, 1952), both alone and in combination with insulin, growth hormone and each other. The effect of administering pancreatic hyperglycemic preparations was also examined. Histologic examination of the animals treated with diethyldithiocarbamate and cobaltous chloride was conducted, together with that of control animals. A discussion of the results will be presented, suggesting that obese animals may be characterized by a hypersecretion both of insulin and of hyperglycemic factor, presumably pancreatic, with growth hormone a tropic factor to the latter hormone.

METHOD

The animals used in these studies, both obese and non-obese, varied in age from 3 to 6 months. The majority of animals used in comparative blood sugar determinations were 3 to 4 months old. The weight of the non-obese animals was in the 17 to 25 gm. range, that of the obese animals in the 40 to 75 gm. range.

All animals were kept singly or by pairs in small screen-bottomed cages. (Keeping obese animals in large cages decreases intakes and drastically reduces non-fasted glucose levels.) Constant environmental temperature (75° F. = 29° C.) and illumination (9 A.M. to 5:30 P.M.) prevailed in the animal rooms. The mice had free access to food (Purina Laboratory Chow) and to water. Glucose determinations were done by the Folin (1930) ferricyanide method on samples of 0.1 cc. of tail blood precipitated by the Somogyi (1945) method. Previous studies have shown that duplicates were unnecessary as the difference between samples is less than 3%. Large scale bleeding is not feasible in these small animals particularly if to be repeated. All chemicals and hormones injected were dissolved in 0.25 cc. of sterile water and administered by the subcutaneous route. For histologic studies animals were sacrificed by decapitation. Pancreases, adrenals, pituitaries and sections of liver were fixed in "formol sublimate" (one part concentrated formalin and 9 parts saturated aqueous mercuric chloride). Sections of pancreas were stained by Gomori's chromalum hematoxylin and aldehyde fuchsin trichrome procedures (1941, 1950, 1950). Aldehyde fuchsin was also used for pituitaries as well as Mallory's aniline blue (Mallory, 1936) and in some instances the McManus-Hotchkiss periodic acid Schiff reaction after Purves and Griesbach (Purves and Griesbach, 1951). Livers and adrenals were stained with hematoxylin and eosin.

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[4] The formula of diethyldithiocarbamate is $(C_2H_5)_2$ NCSSH. The compound will be referred to henceforth as DEDTC. The compound was obtained from the Matheson Co., Inc., East Rutherford, N. J. and from the Paragon testing Laboratories (Orange, N. J.) and recrystallized. Different crude lots appear variable in purity.

RESULTS

A. *Blood glucose.* Table 1 gives results of blood glucose determination conducted on 107 obese and 73 non-obese animals, all born in the stock maintained at the Jackson Memorial Laboratory, Bar Harbor Maine, since July 1952. Obese animals and non-obese animals were treated with 2 mg. of growth hormone[5] for three days. It is readily seen that approximately one-third of the obese animals had blood glucose levels below 140 mg. %. Growth hormone administration doubled the blood glucose levels of these animals, and considerably increased the glucose levels of animals normally hyperglycemic. The hyperglycemeic effect lasted for more than a week. Among the non-obese animals, about 14% showed blood glucose levels above 140 mg. % the highest value recorded being 153 mg. %.

TABLE 1. BLOOD GLUCOSE DISTRIBUTION AND EFFECT OF GROWTH HORMONE ADMINISTRATION (2 MG. ×3) ON NON-FASTED OBESE AND NON-OBESE MICE

	Non-obese (73 mice)			Obese (107 mice)		
Untreated Range (mg. per cent)	<100	100–140	>140	<140	140–200	>200
Percentage distribution	35.9	50.0	14.1	33.7	41.8	24.5
Average and S.D. for the range (mg. per cent)	85 ± 37	118 ± 11	145 ± 4	115 ± 51	164 ± 40	289 ± 55
Average and S.D. after growth hormone	90 ± 6	116 ± 11	130 ± 17	274 ± 68	303 ± 41	485 ± 82

Growth hormone treatment did not increase, and often decreased blood glucose levels, even in the case of animals whose blood glucose levels were in the upper part of the non-obese range.

B. *Effect of diethyldithiocarbamate.* Four dosages of sodium diethyldithiocarbamate (DEDTC) were used for metabolic studies: 0.2, 1, 2 and 15 mg. per animal. Histologic studies were conducted on animals treated with 15 mg. doses, as well as on a few animals given massive doses of 50 and 100 mg.

1. Metabolic effects on non-obese animals. Twelve thin mice were injected with 0.2 mg. of DEDTC. Hyperglycemia (225 ± 35 mg. per cent) was invariably noted after 2 hours and disappeared during the course of the first day, although blood glucose values remained in the upper part of the non-obese normal range (130 ± 5 mg. per cent) for 3 to 5 days. After a small (1 to 2 gm.) weight loss during the 3 day period immediately following injection, the animals regained an average of 3 to 5 gm. during the following fortnight.

Two groups of 12 non-obese animals each were injected respectively with

[5] We are indebted to Armour and Co., and in particular to Dr. Lachat and Dr. Steelman for their generosity in supplying endocrine preparations.

1 and 2 mg. of DEDTC. Four animals died in each group within 2 hours of injection. Hyperglycemia was again noted 2 hours after injection in the surviving animals. Normal blood glucose values were again reached within 3 days after the injection, except for several animals injected with 2 mg. which showed blood glucose values of the order of 150 mg. % for 2-4 additional days. The weight picture was similar to that observed with animals injected with 0.2 mg.

After injection of 15 mg. doses, 8 non-obese mice again showed hyperglycemia 2 hours after administration, with two animals reaching levels of 475 mg. %. High levels lasted for 10 to 13 days before returning to normal

TABLE 2. EFFECT OF A SINGLE DOSE OF DEDTC ON BLOOD GLUCOSE LEVELS OF OBESE HYPERGLYCEMIC MICE

Treatment	No. of mice	Before treatment	24 hrs.	6 days	14 days
0	12	201 ± 30			256 ± 42
0.2 mg.	9	192 ± 36	147 ± 73	141 ± 17	182 ± 47
1 mg.	11	179 ± 26	130 ± 31	156 ± 26	181 ± 32
2 mg.	11	231 ± 57	139 ± 45	161 ± 42	219 ± 68
15 mg.	11	226 ± 24	139 ± 18	150 ± 19	161 ± 29
0, pair fed with animals treated with 15 mg.	8	210 ± 13	233 ± 35	212 ± 18	212 ± 19

values. The weight picture was similar to that observed at the lower dose levels. This dose was found to be the most effective in terms of modifying response to insulin, growth hormone and cobaltous chloride (see below).

2. Metabolic effects on obese animals. Table 2 gives results for 4 typical series of 9, 11, 11 and 11 obese animals respectively injected with doses of 0.2, 1, 2 and 15 mg. of DEDTC. One group of 12 animals was injected with saline. Blood glucose levels previously normally elevated (180 mg. % or above) were down to non-obese normal values within 24 hours. This low level was observed to be reached in a number of animals (12) after a series of maxima and minima occurring during the first 6 hours. Peaks were frequently above 300 mg. %, low values of the order of 50 to 60 mg. %. Blood glucose levels were observed to rise again 4 to 8 days after treatment, reaching values of the order of those prevailing before the injection about 2 weeks after DEDTC administration. Weight losses of 4 to 6 gm. took place during this 2 week period. During the same interval of time, non-treated obese animals gained on the average 7 gm.

That the low blood glucose levels observed after treatment are not simply due to inanition is demonstrated by the fact that paired-fed untreated obese animals exhibited much higher blood glucose levels (Table 2). Paired-fed untreated animals lost little or no weight; they appeared far less active physically than treated animals.

Six obese animals were subjected to repeated treatment. During the week following injection of 2 mg. of DEDTC they lost an average of 4 gm. and stayed at this lower level during a second week. They were then injected with a second dose of 2 mg. and lost an additional 1.6 mg. during this third experimental week. A similar group of animals, left untreated, gained an average of 10 gm. during the same period.

C. *Effect of DEDTC and insulin.* Six untreated 6-month old obese mice were injected with 1 unit of insulin.[6] No decrease in blood glucose was observed within the two hours following treatment. Six similar untreated animals were injected with 20 units of insulin. Blood glucose values were decreased from 285 ± 40 mg. % to 225 ± 45 mg. %. Ten obese animals, in-

TABLE 3. EFFECT OF DEDTC TREATMENT ON THE REACTION OF OBESE HYPERGLYCEMIC MICE TO INSULIN

Number of animals	Treatment	Before treatment	After DEDTC	After insulin	Observations
6	20 U insulin	285 ± 40	—	225 ± 45	
4	2 mg. DEDTC 10 U insulin	175 ± 5	125 ± 9	65 ± 10	
4	5 mg. DEDTC 10 U insulin	187 ± 35	151 ± 21	50 ± 19	
4	10 mg. DEDTC 10 U insulin	233 ± 40	107 ± 14	38 ± 10	2 died
10	15 mg. DEDTC 10 U insulin	169 ± 41	139 ± 64	55 ± 6	10 animals showed convulsion, 5 died

jected 4 days previously with 15 mg. of DEDTC were injected with 10 units of insulin. All showed convulsions within an hour after injection and 5 died. Pretreatment with smaller doses of DEDTC were also effective (Table 3).

D. *Effect of DEDTC and growth hormone.* Three groups of 8 animals were used. One group was injected daily for 3 days with saline, a second group similarly with 0.2 mg. of growth hormone, a third group received 2 mg. of DEDTC the first day and 0.2 mg. of growth hormone daily during the three days. Blood glucose was determined at the end of the 3 day periods. Saline injection had no effect. The animals which had received growth hormone alone showed a marked increase in blood glucose level. DEDTC treatment in the third group eliminated this rise (Table 4).

E. *Effect of DEDTC and exposure to cold.* Six obese mice injected 4 days previously with 1 mg. of DEDTC, and 6 obese untreated animals were placed in a cold room (3° C) at the same time. The average duration of survival of untreated animals was 4 hours, similar to that observed pre-

[6] Squibb's crystalline insulin was used in these experiments.

TABLE 4. EFFECTS OF DEDTC TREATMENT ON THE REACTION OF OBESE-HYPERGLYCEMIC MICE TO GROWTH HORMONE

Number of animals	Treatment	Blood glucose level			
		Before treatment	After saline	After DEDTC	After growth hormone
8	Saline	216 ± 20	192 ± 31	—	—
8	Growth hormone (2 mg. ×3)	193 ± 35	—	—	322 ± 11
8	15 mg. DEDTC 2 mg. ×3 growth hormone	211 ± 77	—	148 ± 31	144 ± 20

viously (Mayer and Barrnett, in press). Of the treated animals, 2 survived until the experiment was terminated (2 days); the average duration of survival of those who died was 8 hours. The animals who survived the two days were those for whom the treatment had been most effective from the points in view of weight loss and blood glucose response.

F. Effect of cobaltous chloride and DEDTC.

1. Effect of cobaltous chloride alone. Fourteen obese animals (average blood glucose levels 159 ± 40 mg. %) were given 2.5 to 10 mg. of cobaltous chloride. All showed extreme hyperglycemia (above 400 mg. %) 2 hours after injection. Only four animals were alive at 24 hours and of these 3 still showed hyperglycemia (of the order of 300 mg. %) and 1 hypoglycemia (67 mg. %). One hyperglycemic animal survived more than 4 days. On that day its blood glucose level was still high (326 mg. %).

Eight obese animals (average blood glucose level 185 ± 30 mg. %) were injected with 1.25 mg. of cobaltous chloride. All survived indefinitely. All showed hyperglycemic levels (above 400 mg. %) 2 hours after treatment; blood glucose levels were still high (250 mg. %) or more in 6 out of 8 animals 24 hours later. Four to 7 days after injection, levels were in the range 130 to 180 mg. %, each one significantly lower than the pretreatment levels. All 8 animals were reinjected 7 days after the first injection. All showed again a rise in blood glucose, although less marked than previously (no higher than 200 mg. %).

Eight non-obese animals similarly treated showed hyperglycemia after 2 hours (from an average of 107 ± 5 to 290 ± 60 mg. %). All failed to survive 24 hours.

2. Effect of cobaltous chloride on DEDTC treated animals. Ten obese animals treated with 15 mg. of DEDTC were injected 10 days later with 1.25 mg. of cobaltous chloride. The range of blood glucose in these animals was 110 to 240 mg. %. No appreciable change was noted in the individual blood glucose levels 2 hours, 24 hours or 4 days after injection.

G. *Effect of pancreatic hyperglycemic preparations.* Six untreated non-obese mice and 12 untreated obese animals were injected with 1 mg. of pan-

creatic "hyperglycemic" preparation.[5] No effort was made to inactivate residual insulin. All non-obese animals treated died within one hour, having exhibited convulsions. Blood glucose levels, determined in 3 animals were below 20 mg. %. By contrast, all obese animals showed rises in blood glucose levels varying from 29 to 84 mg. %.

H. *Histologic results.*

1. Untreated animals. A previous report (Bleisch, Mayer and Dickie, 1952) described the increase in number and the hypertrophy of the islets of Langerhans in the obese mice. In the present study, it was further observed that the islets of Langerhans of obese animals are distinctly deficient in beta granules as compared with non-obese siblings (or with Swiss mice). This deficiency is clearly demonstrable when Gomori's aldehyde fuchsin stain is used; this technic was developed since the first morphologic observations were reported. Besides increase in number, hypertrophy and Beta cell degranulation, the islets of Langerhans of obese animals show a high degree of vascularization and are frequently characterized by the presence of a clear zone between capillaries and adjacent beta cells. The trabecular arrangement of islet cells noted occasionally in the previous anatomic description, was frequently seen in the present study. A thorough study of the variation of the appearance of the pancreases with age in the obese and non-obese animals, will be reported later.

2. Treated animals. Twenty-four mice were injected with 15 mg. of DEDTC for histologic examination. Their age was between 5 and 6 months. The group comprised 6 obese males and 6 obese females, 6 non-obese males and 6 non-obese females. Four animals representing one of each of the four above categories were sacrificed at the following intervals after injection: 1,4 and 12 hours, 2,4 and 8 days. In addition, 4 animals, 2 obese and 2 non-obese were sacrificed 24 hours after receiving 50 mg. of DEDTC per animal. Two animals, 1 obese and 1 nonobese died $4\frac{1}{2}$ to 5 hours after administration of 100 mg. DEDTC. These six animals were also examined histologically. The following observations were made:

A. Non-obese animals. Following administration of 15 mg. of DEDTC, no changes were observed in the appearance of either the beta cells or the alpha cells at the various times after treatment. In the one animal dying after administration of the 100 mg. dose, marked vacuolization of all pancreatic cells was observed, acinar as well as islet, with apparent loss of alpha and beta granules. No significant changes were seen in adrenal, liver or pituitary tissues of any of the non-obese animals.

B. Obese animals. Following administration of 15 mg. of DEDTC, marked changes were observed in the beta cells of obese animals sacrificed 4, 12 and 48 hours after injection. A striking increase in number of beta granules was observed, to the extent that the individual beta cells were no

longer distinguishable from those of the non-obese siblings. Two of the three animals treated with 50 to 100 mg. doses showed the same changes when sacrificed 4–24 hours after injection. Four days after treatment the appearance of the beta cells reverted to that preceding treatment, i.e., again showing deficient beta granulation. No significant changes were observed in alpha cells, livers, adrenals, or pituitaries.

A few obese animals treated with 25, 50, and 100 mg. of cobaltous chloride were also examined. At the lower dosage there was again apparent regranulation of the beta cells.

DISCUSSION

The islets of Langerhans of the untreated obese mice have a highly distinctive appearance as compared with those of non-obese siblings. Their increased size and number, their rich capillary blood supply (which gives the pancreases, on dissection, a pinker appearance than that of the non-obese animals), the trabecular arrangement of the beta cells, the striking beta cell degranulation all bespeak an increased functional activity. It is tempting to interpret the hyperplastic degranulated beta cells as evidence of excessive insulin demand, consistent with the insulin resistant hyperglycemia exhibited by these animals. In view of the increased size and number of islets of the obese animals, it is impossible at this time to compare the total number of beta granules and hence presumably the total insulin content present at a given time in the pancreases of obese and non-obese animals. (Direct determination of the insulin content of the pancreas of the two types of siblings is being pursued at the present time in collaboration with G. A. Wrenshall, Banting and Best Institute, University of Toronto.)

The metabolic and morphologic data suggest that obese mice secrete in excessive amounts a hyperglycemic "anti-insulin" factor, the tropic hormone to which is growth hormone and that DEDTC treatment eliminates temporarily this hypersecretion. In obese animals which do not show non-fasted high glucose levels, hyperglycemia is immediately elicited by growth hormone treatment, in opposition to what is observed in non-obese mice. This finding indicates that hyperglycemia or capacity for growth-hormone-induced hyperglycemia is a universal finding in the obese mice and justifies speaking of the syndrome as the obese-hyperglycemic syndrome. There is no basic genetic separation between the obesity and the hyperglycemia, although additional genetic factors may intervene to delay or "mask" the non-fasted hyperglycemia until revealed by growth hormone assay. Possibly a genetically determined capacity for increasing insulin production to a greater extent is responsible for the "masking" effect. DEDTC treatment eliminates the capacity of the organism to respond by hyperglycemia

to growth hormone treatment. Results of administration of beef pancreatic extracts would indicate that obese mice contain enough hyperglycemic factor to be insenitive to the insulin contaminant while still able to respond to additional exogenous hyperglycemic factor.

It is not possible on the basis of the data presented in this study alone to relate the production of this factor specifically to either the alpha or the beta cells of the islets of Langerhans, both of which types are increased in number in apparently proportional amounts in the obese mice. The observations obtained with cobaltous chloride are not easily interpreted at the present time. Extrapolation from the results obtained by the Japanese workers on rabbits would, of course, tend to designate the alpha cells as the secretory elements corresponding to this endocrine factor. It should be mentioned again that to date we have seen no significant alpha cell changes following DEDTC treatment. Additional work will doubtless bring needed light on this question. At any rate, the regranulation of the beta cells following DEDTC or cobalt treatment could indicate that a constant drain on insulin reserves is no longer operative when secretion of the hyperglycemic factor is interrupted.

Finally, from the point of view of the etiology of the obese hyperglycemic syndrome it still has to be shown whether the hypersecretion of this postulated hyperglycemic factor is an adaptive adjustment to the metabolic block to acetate oxidation previously demonstrated (Guggenheim and Mayer, 1952) or whether, as the authors would tend to believe at the present time, an increased insulin secretion, responsible for the acetate block and increased lipogenesis is a result of the tendency of the pancreas to correct for the primary hypersecretion of the hyperglycemic factor. It is hoped that further work will also cast light on this important question.[7]

SUMMARY

1. A certain proportion of mice with the obese-hyperglycemic syndrome show normal non-fasted blood glucose levels. Hyperglycemia can be invariably elicited in these animals by growth hormone administration, to which non-obese animals are resistant. This suggests the presence in the stock of a genetic factor responsible for the "masking" of the hyperglycemia. Impure pancreatic extracts cause hyperglycemia in obese mice, hypoglycemia in non-obese animals.

2. Diethyldithiocarbamate treatment eliminates for a period of one to

[7] Results of the cooperative determinations of pancreatic insulin content conducted with Dr. G. A. Wrenshall, Banting and Best Institute, University of Toronto, indicate that the pancreas of mature obese-hyperglycemic mice does in fact contain several times more insulin than that of comparable non-obese animals.

two weeks, the hyperglycemia, insulin resistance and response to growth hormone of obese hyperglycemic animals. These effects are not simply due to reduction of food intake, although this phenomenon and weight loss do take place. Diethyldithiocarbamate treatment also prevents the hyperglycemia which normally follows cobaltous chloride injection. From a histologic standpoint, the untreated obese animals show deficient beta cell granulation in the islets of Langerhans and diethyldithiocarbamate temporarily restores beta granulation in these animals to levels comparable with non-obese siblings.

3. An interpretation of the results in the context of an increase in secretion of the two pancreatic hormones (both insulin and a hyperglycemic factor) is suggested.

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EFFECT OF ADRENAL CORTICAL EXTRACT, CORTISONE AND HYDROCORTISONE UPON THE WORK OF THE ADRENALECTOMIZED-EVISCERATE RAT

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CONSIDERATION has been given to the hypothesis that cortisone must be chemically modified in the liver before it manifests hormonal activity (Ralli, 1952). The data of the present study show that adrenal cortical extract, cortisone and hydrocortisone are each capable of increasing the work output of the adrenalectomized-eviscerate rat.

METHODS

Male rats of the Sprague-Dawley strain from the Upjohn breeding colony were maintained on a diet of Archer Dog Pellets. The technique of evisceration, which is done in two stages, has been described (Ingle, 1949). When the animals reached 250 ± 2 gm. they were anesthetized (intraperitoneal injection of 18 mg. of cyclopal sodium) and were subjected to the second stage of the operation in which the adrenal glands were removed with the other intra-abdominal organs. The stimulation of muscle was started immediately following evisceration. The procedure was according to Ingle (1944) with the following modifications. A Nerve Stimulator, Model B, Upjohn, was used to stimulate muscle 5 times per second by a D.C. pulse of 20 ma. having a duration of 20 msec. The gastrocnemius muscle of the left hind leg was weighted with 100 gm. The distance the weight was lifted was registered on automatic work recorders. Each recorder revolution represented approximately 400 gram-centimeters of work.

Each rat received, by continuous intrajugular injection, 20 cc. per 24 hours of a solution containing glucose, 4 units of regular insulin (Lilly), 10,000 units of penicillin and 5 mg. of streptomycin with and without adrenal cortical extract (ACE) or steroids. The ACE represented 20 glycogen units (Pabst, Sheppard and Kuizenga, 1947) per rat per 24 hours and doses of 2 mg. of hydrocortisone and 4 mg. of cortisone (nonesterified) per rat per 24 hours were used. These doses each have an optimal effect upon the ability of the adrenalectomized rat to work (Ingle, 1953).

The animals were enclosed in a cabinet with temperature constant at $28 \pm 0.5°$ C. The infusions were started simultaneously with the stimulation of muscle immediately following the operation.

EXPERIMENTS AND RESULTS

In experiment one, 49 adrenalectomized-eviscerate rats were each given 20 cc. of solution per 24 hours containing insulin, antibiotics (see METHODS) and a glucose load of 100 mg. per 100 gms. of rat per hour. Twelve of the

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rats each received the above solution without the addition of hormones; 14 rats each received 20 glycogen units of ACE per 24 hours added to the above solution; 12 rats each received 2 mg. of hydrocortisone per 24 hours added to the above solution; and 11 rats each received 4 mg. of cortisone per 24 hours added to the above solution. The average work output of the rats in each of the three treated groups was significantly greater than that of the untreated group. For example, the average difference between the

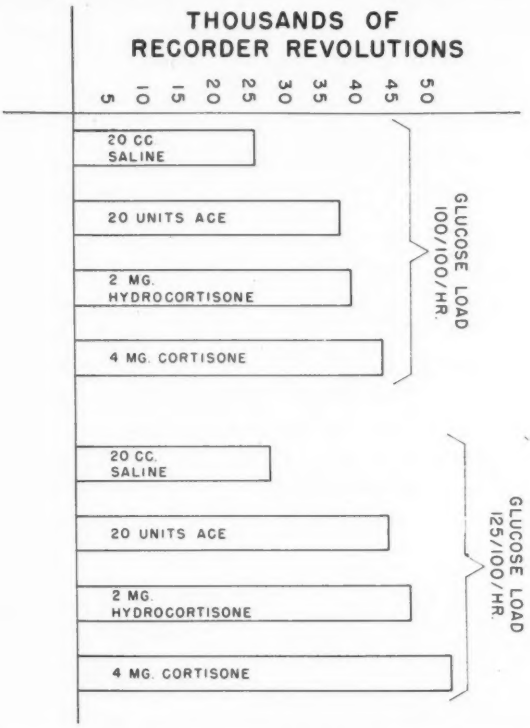

FIG. 1. Work output of adrenalectomized-eviscerate rats treated with adrenal cortical hormones. Averages for experiments 1 and 2.

saline and the ACE groups was $12{,}013 \pm 3876$ recorder revolutions. Although the average work output for the rats treated with hydrocortisone and with cortisone was greater than in the ACE group, the differences among the three treated groups failed to meet the usual requirements for statistical reliability. For example, the average difference between the ACE and the cortisone groups was 5833 ± 4763 recorder revolutions.

Experiment 2 was identical with experiment 1 except that a glucose load of 125 mg. per 100 gms. of rat per hour was used in 63 adrenalectomized-eviscerate rats. Fifteen rats received the solution without the addition of

hormones; 18 rats each received 20 glycogen units of ACE per 24 hours added to the above solution; 15 rats each received 2 mg. of hydrocortisone per 24 hours added to the above solution; and 15 rats each received 4 mg. of cortisone per 24 hours added to the above solution. The results were similar to those of experiment 1. The average work output in each of the three treated groups was significantly greater than that of the untreated group. The difference between the untreated and the ACE-treated groups was $16{,}865 \pm 4467$ recorder revolutions. Again the average work output of the rats treated with cortisone and hydrocortisone was greater than for the ACE-treated group but the differences among the three treated groups failed to meet the usual requirements for statistical reliability. The average difference between the ACE and the cortisone groups was 5968 ± 4563. The data of experiments 1 and 2 are summarized in Figure 1.

DISCUSSION

It was previously shown that adrenalectomized-eviscerate rats treated with ACE perform significantly more work than do untreated animals (Ingle, 1938). The data of the present experiments show that cortisone and hydrocortisone are also biologically active in the absence of the liver and other intraabdominal organs. These results do not exclude the possibility that the liver affects the biologic activity of these steroids when it is present. It has been shown by a number of laboratory and clinical studies that cortisone and hydrocortisone have local effects upon tissues which presumably do not require mediation by other tissues (for review see Ingle and Baker, 1953).

SUMMARY

Adrenalectomized-eviscerate rats were given continuous intravenous injections of solutions containing glucose, insulin and antibiotics with and without adrenal cortical hormones. Adrenal cortical extract, cortisone and hydrocortisone each enhanced the work output above the values for untreated rats thereby showing that modification of these steroids in the liver is not essential for the manifestation of biologic activity.

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